MATERNAL-EMBRYONIC INTERACTIONS DURING EARLY PREGNANCY IN CATTLE

By

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Dedicated to Mario Rodolpho Giovanni Binelli (late) and Arnaldo Monteiro de Oliveira (Grandfathers), Guilherme Jose Binelli (Father), Ricardo Binelli (Brother), Luiz Alberto de Oliveira (Uncle), Cicero Spiritus, Paul Campbell, Zilmar Ziller Marcos, H. Allen Tucker and William W. Thatcher (Mentors)

To my family and friends

and

To an enlightened humankind

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MATERNAL-EMBRYONIC INTERACTIONS DURING EARLY PREGNANCY IN CATTLE

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Maternal-embryonic physiological communications are an important feature of processes in the reproductive cycle. Communications occurring during oviductal transit of gametes/embryos and during maternal recognition of pregnancy for maintenance of the corpus luteum (CL) were studied in cattle. Objectives were 1) to study the distribution pattern of oviductal secretory proteins secreted by cows bearing persistent or fresh dominant follicles (PDF or FDF, respectively); 2) to examine the signal transduction system stimulated by bovine interferon-τ (bIFN-τ) in endometrium; and 3) to characterize the effects of bIFN-τ on prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) production by bovine endometrial (BEND) cells. Presence of PDF reduces fertility in cattle. Proteins synthesized from

infundibulum, ampulla and isthmus from oviducts ipsilateral and contralateral to CL of cows bearing PDF or FDF were examined by two-dimensional fluorography. Presence of PDF altered distribution of secretory proteins in a side- and region-specific manner. Changes in the oviductal environment may contribute to decreased fertility of cows bearing a PDF. Conceptus-produced bIFN-τ suppresses endometrial $PGF_{2\alpha}$ pulses in vivo, and is required for maintenance of pregnancy. The hypothesis was that bIFN-τ stimulated synthesis of endometrial proteins through the jak kinases (JAK)-signal transducer and activator of transcription (STAT) pathway of signal transduction. Presence of and bIFN-τ-induced tyrosine phosphorylation of STAT proteins were demonstrated via immunoprecipitation (IP) and immunoblotting (IB) techniques, while bIFN-τ-induced secretory proteins were measured by fluorography in endometrial explants obtained from day 15 cyclic cows. BEND cells were used for remaining experiments. Presence of STATs, tyrosine-phosphorylation, dimer complex formation and nuclear translocation were measured through IP, co-IP and IB. Binding of activated STAT complexes to cis-acting elements present in the regulatory region of interferon-inducible genes was determined using electrophoretic mobility shift assays. Bovine IFN-τ stimulated synthesis of interferon-regulatory factor-1 (IRF-1) in BEND cells as determined by IB. The bIFN-τ regulates synthesis of phorbol 12,13 dibutyrate (PDBu)-induced $PGF_{2\alpha}$, as measured through radioimmunoassays (RIA). Regulation was associated

with bIFN-τ -induced suppression of phospholipase-A_2 and cyclooxygenase-2 protein expression and enzymatic activity measured through IB and RIA. Collectively, experiments elucidated mechanisms that are involved in maternal-conceptus crosstalk required for successful reproductive outcome.

CHAPTER 1
INTRODUCTION

The world's human population is increasing at a fast rate, and consequently the need for basic nutrients, including carbohydrates, lipids, proteins and minerals, is also increasing. Animal agriculture historically has been one of the most important sources of nutrients for humans. Dairy cows efficiently metabolize feed nutrients and synthesize milk, which provides protein and energy in a suitable form for human consumption. Lactation is a final step in the reproductive cycle which is dependent on successful production of viable gametes, conception, pregnancy and parturition. First insemination conception rates for dairy cattle is 52 to 57% (Mawhinney and Roche, 1978). Improvements on such rates will be required for dairy products to continue being a viable source of nutrients for humans.

Classically, study of animal performance has focused on genetic and environmental effects on a given production trait, such as meat or milk production. Reproduction poses an interesting scenario, in which reproductive processes are modulated by the interactions between both the maternal and embryonic genomes. Moreover, reproductive processes are influenced by external environment, but more importantly, by the internal environment (i.e., the

reproductive tract). Both maternal and embryonic units influence such internal environment, and physiological crosstalk is a hallmark of the process. As a result, a complex set of coordinated interactions takes place and it is these interactions that will dictate a successful reproductive outcome. My thesis is that failure in maternal-embryonic communications leads to decreased reproductive rates.

This dissertation examines the role of maternal-embryonic communications during two physiological windows of the reproductive cycle and their importance on the overall reproductive process. Chapter 3 describes steroid hormone-modulation of protein synthesis and secretion in the oviduct, where final stages of gamete maturation, fertilization and early embryo development occur. Chapters 4, 5, 6 and 7 characterize changes in intracellular and secretory processes of the maternal endometrium in response to a conceptus-secreted factor, interferon-τ, that is essential for maintenance of pregnancy.

A greater understanding of the basic mechanisms regulating reproductive processes, specially mechanisms involving maternal-embryonic interactions, is necessary to improve conception rates in cattle.

CHAPTER 2
LITERATURE REVIEW

Maternal-embryonic Communication as a Requirement for Successful Pregnancy

Communications of a physiological nature are very common between embryonic (and pre-embryonic) and maternal units from the development of the oocyte (i.e., before fertilization; Eppig et al., 1997a) to parturition (Bazer and First, 1983; Fuchs and Fields, 1999) and lactation (Thatcher et al., 1980). The maternal unit constitutes all tissues in the female reproductive tract that directly or indirectly interacts with gametes or conceptus (conceptus = embryo and associated embryonic membranes). Appropriate exchange of hormonal signals between the two units is required for successful establishment and completion of several windows of the reproductive cycle. Moreover, each window requires unique signals that have been studied in detail and that have unique implications on the outcome of the reproductive process (i.e., live, viable offspring). A general review of the literature on several critical windows on the reproductive cycle will be presented. A more detailed review will be offered on embryonic-maternal interactions during the time of embryo transit through the oviduct and during the window of maternal recognition of pregnancy occurring at around day

17 of pregnancy. The focus of this review will be on the bovine species, but data from other species will be presented whenever appropriate.

Communications Between Gametes and Maternal Units

Gametes are differentiated cells with the specific function of conveying genetic information from each paternal and maternal unit to a zygote during the process of fertilization. To ultimately undergo fertilization, both paternal and maternal gametes interact with somatic cells in maternal reproductive tissues.

Egg-oocyte. During development of follicles, oocytes change the program of granulosa cells in the follicle, so that the default program of mural granulosa cell differentiation is suppressed. As a consequence, cells surrounding the oocyte become more specialized in functions that favor development of the oocyte. In mature Graafian follicles, this layer of differentiated granulosa cells forms a small pedicle of cells, the cumulus oophurus, which contains the oocyte and protrudes towards the interior of the antrum. Eppig and coauthors (1997a) hypothesized that oocytes control their own microenvironment by regulating differentiation of the supporting cells that are in direct communication with them. For example, expression of luteinizing hormone (LH)-receptors in cumulus cells is abrogated by presence of the oocyte (Eppig et al., 1997b).

Oocyte-oviduct. In cattle, the oviduct is divided in three functional regions, namely infundibulum (INF), ampulla (AMP) and isthmus (IST; Hafez, 1993a). The INF is opened to the peritoneal cavity of the body, the IST connects the

oviduct with the uterus, and the AMP is localized between INF and IST. The initial interaction of the oocyte with the oviduct takes place at the INF. The INF "picks up" the ovulated oocyte and initiates its transport towards the uterus. Overall flow of oviductal fluid is towards the body cavity (Hafez, 1993a). Therefore, in order for the oocyte to be transported to the site of fertilization (AMP), it must interact with ciliated epithelial cells lining the oviductal lumen. Balance between oviductal fluid flow and ciliary beating towards the uterus yields a net movement of the oocyte towards the AMP and ultimately, uterus. During this trajectory towards the site of fertilization, the oocyte is under the influence of products secreted by the oviduct which could modulate its development and the process of fertilization (Buhi et al., 1997a). For example, Kouba and coworkers (1999) determined that a major secretory protein of the AMP, named POSP (porcine oviductal secretory protein), has a role in decreasing occurrence of polyspermy during fertilization in pigs. This supports the findings of Nancarrow and Hill (1995) that an estrus-associated glycoprotein, a protein homologous to POSP in sheep, increased blastocyst formation after in vitro fertilization. Furthermore, Staros and Killian (1998) identified six proteins in oviductal fluid, including a POSP-like protein that are associated with bovine oocytes in vitro.

Sperm-oviduct. Austin (1951) and Chang (1951) independently reported that freshly ejaculated rat and rabbit spermatozoa were incapable of penetrating an oocyte. The ability of fertilization was only acquired after the sperm spent a period of time in the female reproductive tract, a process called sperm

capacitation. These early findings supported the idea of the necessity of interaction between male gametes and products of the oviduct prior to fertilization. In fact, incubation of sperm cells with oviductal fluid capacitated and sustained sperm mobility in vitro (Parrish et al., 1989; McNutt and Killian, 1991). Moreover, Boatman and Magnoni (1995) identified and purified an oviductal factor (oviductin) that acts to enhance sperm penetration in follicular oocytes.

Communications Between Conceptus and Maternal Units

Conceptus-oviduct. Following fertilization, the conceptus continues to interact with the AMP and IST before it reaches the uterus. During this period, the conceptus undergoes initial cell divisions, and there is a possibility of a continual influence of oviductal products on the conceptus. For example, Buhi and coworkers (1993) showed that gold particles immunoreactive with porcine oviductal secretory protein are associated with flocculent material in the perivitelline space surrounding the blastomeres and in the zona pelucida of embryos from the four-cell stage to blastocysts. Moreover, semi-purified oviductal specific protein improved cleavage rates of embryos fertilized and developed in vitro (Hill et al., 1996). In contrast, at least in pigs, presence of gametes or embryos did not affect production of specific oviductal proteins, suggesting that a regulation of oviductal function by gametes or embryos is probably not important during this stage of the reproductive cycle (Buhi et al., 1990).

A series of growth factors have been identified in the oviduct during the estrous cycle and early pregnancy in different species (summarized in Buhi et al., 1997a). For example, Paula-Lopes and coworkers (1999) reported synthesis and secretion of interleukin-1β both in oviduct and uterus of cyclic cows. Moreover, interleukin-1β stimulated in vitro development of embryos when added before day 5, which suggests a oviductal effect (Paula-Lopes et al., 1998). Although limited research has been performed to date to elucidate specific roles of growth factors in fertilization and early embryo development, it is possible that growth factors act in an autocrine and paracrine fashion to influence these processes (Chegini, 1996).

Conceptus-uterus. In cattle, it takes 72 to 84 h from the time of ovulation to the time embryos enter the uterus (Betteridge and Flechon, 1988). From entry into the uterus to parturition there are multiple examples of interactions between the conceptus and the maternal unit, which will be discussed elsewhere in this review. A striking example of such interactions is the process of maternal recognition of pregnancy associated with maintenance of the CL, which will also be presented in detail afterwards. Briefly, maintenance of pregnancy requires a steady supply of progesterone (P_4) from the corpus luteum (CL). In contrast, in cycling animals, it is necessary that P_4 concentrations decrease so that animals can return to estrus. This decrease is accomplished in response to pulses of uterine prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$) that have lytic actions on the CL. Uterine

physiology must be changed to suppress this pulsatile release of $PGF_{2\alpha}$ for the conceptus to survive. The conceptus-secreted interferon-τ (IFN-τ) interacts with the uterine tissue to decrease production of $PGF_{2\alpha}$, thereby allowing for maintenance of CL and consequent sustained elevated P_4 concentrations.

The process of luteolysis includes the action of follicular estrogen on a P_4-primed uterus, which is capable of secreting $PGF_{2\alpha}$. Driancourt and coworkers (1991) formulated the hypothesis that one possible aspect of the antiluteolytic mechanism induced by conceptus could involve attenuation in development of follicles on the ovary adjacent to the pregnant uterine horn. They determined that the number of follicles greater that 7 mm was reduced in the ovary containing the CL after day 22 of pregnancy. Moreover, size of the largest follicle was greater on the ovary contralateral to the pregnant uterine horn. An additional study comparing follicular development in pregnant versus hysterectomized cows indicated that products of pregnancy, either secreted directly or induced by the conceptus, decreased intraovarian follicular development in a local manner (Thatcher et al., 1991). This could enhance embryonic survival by attenuating luteolytic mechanisms (Thatcher et al., 1994b).

The examples above illustrate the common theme of maternal-gametic and maternal-embryonic interactions, and their occurrences throughout the reproductive cycle. Failure of appropriate communication between maternal and embryonic units can lead to disruption of the reproductive cycle and termination of pregnancy. Next I will examine the issue of embryonic mortality in cattle. In

later sections I will discuss the implications of a failure in maternal-embryonic cross-talk on embryonic mortality.

Problems Associated with Fertilization Failure and Embryonic Mortality in Cattle

Susceptible Periods During Pregnancy

Calving rates to a single insemination are reported to be 52 to 57% for dairy cows (Mawhinney and Roche, 1978) despite fertilization rates of about 89% (Henricks et al., 1971). Diskin and Sreenan (1980) utilized beef heifers to determine embryo survival during discrete periods within pregnancy. They reported up to 93% survival rates to day 8, 66% to day 16 and 58% to day 42. These data indicate that minor losses are due to fertilization and embryonic death before day 8, which encompasses the period of embryo permanence in the oviduct and development to the blastocyst stage in the uterus. However, in a group of infertile cows, there are appreciably greater losses (~40%) due to failure of fertilization (Tanabe and Cassida, 1949) and additional losses by day 35 (40%; Ayalon 1978). In normal cows, a large percentage of embryos is lost between days 8 and 16 of pregnancy, which is the period of embryonic elongation and maternal recognition of pregnancy associated with CL maintenance. The substantial losses of pregnancies during the first 16 days of pregnancy has obvious economic impacts in the livestock industry, and represent an opportunity for animal scientists and reproductive physiologists to

improve calf crops. This prompted a great quantity of research to understand mechanisms involved in the processes of fertilization failure and embryonic mortality. Moreover, understanding the mechanisms underlying these alterations in reproductive development will provide the basis for creation of technologies aimed to attenuate fertility problems in the field.

Causes of Fertilization Failure and Embryonic Mortality

Inadequate embryo unit. Both genetic and environmental effects can account for early embryonic deaths. There is an estimated 7.5% death of early bovine embryos because of occurrence of chromosomal abnormalities (Wilmut et al., 1986). Such abnormalities may be inherited or arise during meiosis, fertilization or early cleavage stages (King, 1985).

Environmental effects such as heat stress also decrease the ability of embryos to develop properly. For example, Putney and coworkers (1989) exposed superovulated heifers to hyperthermic conditions for 10 hours after the onset of estrus. There was no difference in the rate of fertilization between heat-stressed and non-heat stressed heifers (control). However, only 12% of embryos recovered from stressed heifers were normal vs. 69% of embryos recovered from control heifers. Since the period of heat stress was administered prior to ovulation and fertilization, it was hypothesized that the detrimental effect of the heat stress was exerted on the oocyte within the follicular environment. In an attempt to investigate the effects of heat stress on embryonic survival from

the time of ovulation to 7 days after estrus, Putney and coworkers (1988a) kept heifers either in thermoneutral or hyperthermic conditions starting 30 hours after onset of estrus. They found similar results to the ones described above, where heat stress increased the proportion of abnormal embryos compared to controls. To pinpoint critical stages of susceptibility of embryos to elevated maternal temperatures, Ealy and coworkers (1993) submitted cows to heat stress on days 1, 3, 5 or 7 of pregnancy. Embryos recovered on day 8 were compared to embryos from cows not heat stressed. Only heat stress at day 1 caused decreased development. Collectively, data from these three heat stress experiments suggest that environmental factors can affect embryo development in multiple stages. Alternatively, it is possible that the toxic effects of heat stress are exerted in the oviductal and uterine environments, which could become sub-optimal for fertilization and embryo development, resulting in abnormal embryos. As a final example of environmental effects on embryo development, Putney and coworkers (1988b) incubated day 17 conceptuses at normal (39 C, 24 hours) or high (39 C, 6 hours; 43 C, 18 hours) temperature regimens and measured de novo protein synthesis by these conceptuses. They found that heat stress not only decreased overall protein synthesis, but more importantly, decreased secretion of IFN-τ. This indicates that under the influence of heat stress, embryos are less capable to sending appropriate signals to the uterus, which are required for maintenance of an environment conducive to pregnancy.

Inadequate maternal unit. As mentioned above, results from the work of Putney and coworkers (1988a; 1989) suggest that the elevated number of abnormal embryos in heifers that underwent heat stress could be a result of the effect of high temperatures on the maternal unit, and not a direct effect on the embryo. In that regard, embryos produced by in vitro maturation/fertilization techniques that are exposed to elevated temperatures (40.5 C) for 12 hours had development comparable to that of controls (56% blastocyst formation; Rivera, Lopes and Hansen, personal communication), supporting the concept that heat stress effects on maternal units may create a toxic environment that is conducive to development of abnormal embryos. Moreover, in the experiment of Putney and others (1998b), they incubated endometrium explants removed from cows at day 17 of the estrous cycle at 39 or 43 C as described above, and measured secretion of $PGF_{2\alpha}$ in the medium. Heat stress caused a pronounced increase in $PGF_{2\alpha}$ production over time compared to controls. This finding suggests that heat stress favors luteolysis and consequent loss of pregnancies.

Measurements of P_4 concentrations in milk following insemination of dairy cows revealed that inseminated-pregnant cows had slightly higher P_4 compared to inseminated-non-pregnant cows (Lamming et al., 1989). This finding prompted the hypothesis that luteal insufficiency could be a cause of increased embryonic mortality in lactating dairy cows. Possible causes of decreased luteal function include (1) poor development of the ovulatory follicle, resulting in a low quality CL (i.e., low weight and consequent low P_4 secretion) and (2) insufficient

luteinizing hormone (LH) support of continuous luteal P_4 secretion. Strategies to provide supplemental P_4 through administration of exogenous P_4 (Van Cleef et al., 1996), use of human chorionic gonadotropin (hCG; Schmitt et al., 1996a), gonadotropin releasing hormone (GnRH) injections (Schmitt et al., 1996b), and GnRH implants (Ambrose et al., 1998) have increased circulating concentrations of P_4 but yielded mixed results on pregnancy rates.

Asynchrony between embryonic and maternal units. Embryo survival may be impaired because of failure in some aspects of the relationship between the embryonic and maternal units, despite the fact that both are normal (Wilmut et al., 1986; Thatcher et al., 1994b). An example of such failures is lack of synchrony between uterus and embryo. During early pregnancy, embryo development depends upon a sequence of changes in the uterine secretions, which in turn is dependent on progressive changes in the maternal hormonal milieu. This phenomenon became established when it was observed that embryos transferred between animals that were not in estrus at the same time caused abnormal development and death of the embryo (Wilmut and Sales, 1981). Moreover, when cows were treated with P_4 from days 1 to 5 of the estrous cycle and received a day-8 embryo on day 5, pregnancy was maintained, indicating that uterine development had been advanced as a result of the exogenous P_4 (Geisert et al., 1991). A condition that can cause asynchrony is exposure of cows to heat stress. Biggers and others (1987) determined that high environmental temperatures between days 8 and 16 of

pregnancy caused a 50% reduction in weight of conceptus compared to control cows. These retarded embryos may not be able to send the appropriate antiluteolytic signals to the maternal endometrium, thereby allowing luteolysis and consequent loss of pregnancy to occur.

Inadequate manipulations of the system. As a means to improve conception rates in livestock operations, researchers have developed several management practices that often include manipulations of the endocrine system of animals. Such practices may sometimes yield unexpected results, including decreased fertility due to disruption of appropriate maternal-embryonic communications. One widespread practice is synchronization of estrous cycles. Estrus synchronization systems are used for artificial insemination, timed insemination and embryo transfer. Most commonly, synchronization is achieved with combinations of treatments with $PGF_{2\alpha}$, progestins and gonadotropin releasing hormone (GnRH; Thatcher et al., 1996). Synchronization with progestins is based on the principle that exogenous progestins, such as progesterone delivered by a controlled internal drug release (CIDR) device, can maintain a sub-luteal concentration of progestin in blood during a period which permits CL regression. In the absence of a CL, removal of the progestin source will result in a synchronized estrus (Macmillan and Peterson, 1993). However, sub-luteal concentrations of progesterone increase LH pulse frequency, which stimulates continuous growth of a dominant follicle (Cooperative Regional Research Project, NE-161, 1996; Savio et al., 1993a; Savio et al., 1993b). This

"persistent" dominant follicle (PDF) is estrogenic, and subsequent fertility, as measured by conception rate at first service (number of pregnancies / number of animals inseminated), is lower compared to animals bearing normal DFs [37.1% vs. 64.8% in heifers, (Savio et al., 1993b); 23.6% vs. 58.2% for cows and heifers (Cooperative Regional Research Project, NE-161, 1996). Possible explanations for reduced fertility include alterations in the oocyte and /or in the oviductal environment. In a study by Ahmad et al. (Ahmad et al., 1995), embryos recovered at Day 6 of pregnancy from cows bearing PDF were less developed (i.e., were less able to reach the 16-cell stage) than embryos from cows ovulating a fresh dominant follicle (FDF). In addition, Revah and Butler (1996) showed that oocytes recovered from PDF showed expanded cumulus cells and condensed chromatin dispersed in their ooplasm. In contrast, compact cumulus cells and intact germinal vesicles were found in oocytes from FDF. Thus, the PDF may induce premature oocyte maturation and/or alter oviduct function, which could affect early embryonic development and decrease fertility. Processes of sperm capacitation, fertilization and early embryonic development in this altered oviductal environment can contribute to decreased fertility experienced by cows developing persistent follicles.

Oviductal Function and Reproductive Failure in Cattle

The Oviduct Environment

The oviduct environment can be simplistically described as presenting physical and chemical characteristics which are conducive to the reproductive processes occurring within the oviduct. Physical and chemical characteristics are described below in the sub-sections "Functional anatomy and morphology" and "The oviduct fluid", respectively. To exemplify the functions of these characteristics, Rieger and others (1995) examined development of embryos in vitro, either in coculture with oviductal cells (both physical and chemical influences) or in serum-free medium pre-conditioned by oviductal cells (chemical influences, only). In both systems, embryos reached the 4-cell stage in 48 hours. However, embryos developing in the coculture system reached the blastocyst stage 24 h before the others and also had significantly more cells. There was no treatment where only physical factors were present, but the conclusion from their data is that probably both physical and chemical characteristics are necessary for best embryo development.

The ovarian cycle. There is a close association between oviduct function and concentrations of circulating ovarian steroid hormones. Therefore, it is appropriate to describe the changes occurring in such hormones during the

estrous cycle. Other aspects of the estrous cycle will be discussed elsewhere in this chapter.

The ovarian cycle consists of cyclic growth and demise of two ovarian structures, the follicle and the CL. Considering one estrous cycle the period comprised between two ovulations, two to three follicular waves of dominant follicle growth occur (Savio et al., 1988; Sirois and Fortune, 1988). Each follicular wave is comprised of periods of recruitment, selection, dominance and turnover or atresia. The ovulatory follicle generated in the last wave does not turn over, but ovulates. The main steroid secretory product from follicles are estrogens, such as E_2. There is a positive relationship between size of follicles and E_2 concentration in the circulation. Since maximum growth of follicles occurs during the dominance phase, the peri-ovulatory period is characterized by highest concentrations of circulating E_2 during the estrous cycle. The ovulated follicle undergoes functional and structural changes to form a CL. The CL grows at a rapid rate to reach a maximum size in about 11 days, remains at its maximum size until about day 16 of the estrous cycle and then regresses (the process of luteolysis). Parallel to changes in CL size are changes in secretion of luteal P_4. Turnover of the dominant follicle is associated with high concentration of P_4, typical of mid-cycle. In contrast, final differentiation and growth of the ovulatory follicle prior to ovulation only occurs in a low P_4 environment.

Functional anatomy and morphology. The oviducts are suspended in the mesosalpynx, a peritoneal fold of the broad ligament. As mentioned earlier, the

oviduct can be divided into three functional regions: the funnel shaped abdominal opening near the ovary INF, which terminates in the fringe-like fimbriae; the more distal dilated AMP and the IST, the narrow proximal portion of the oviduct, connecting to the uterus (Hafez, 1993a). The oviduct can be simply described as a muscular tube with a mucosal lining. There are two muscle coats: an external longitudinal and an internal circular coat (Leese, 1988). Thickness of the musculature increases from the ovarian to the uterine end of the oviduct. Muscular contractions function to mix oviductal contents, aid sperm transport, help denudate the egg, promote fertilization and regulate egg transport. Patterns of oviduct muscular contractions vary with the stage of the estrous cycle, indicating hormonal regulation of this process. Before ovulation, contractions are gentle, but become more vigorous at ovulation. Muscular contractions in the ovarian direction are more common than in the uterine direction (Hafez, 1993a). The oviductal mucosa possesses characteristic folds, with high, branched folds in the AMP and decreasing heights towards the IST to become low ridges. The mucosa consists of one layer of columnar epithelial cells, underlined by a submucosa containing smooth muscle fibers and connective tissue. The oviductal epithelium contains both ciliated and non-ciliated, secretory cells. Ciliated cells are most abundant in the INF and least in the IST. Rate of cilia beating is affected by levels of ovarian hormones, with maximal activity occurring at the periovulatory period. Cilia beating is synchronized and toward the uterus. The opposite direction of coordinated cilia

beating (towards the uterus) and oviduct muscular contractions (towards ovary) maintain eggs in constant rotation, which is essential for fertilization and to prevent oviduct implantation (Hafez, 1993a). Non-ciliated epithelial cells have primarily a secretory function. They contain secretory granules at their apical aspect, and these accumulate during the follicular phase of the estrous cycle, and are released into the lumen after ovulation (Murray, 1992). Treatment of ovariectomized sheep with E_2 stimulates hypertrophy of secretory organelles and accumulation of granules in non-ciliated cells of the AMP (Murray, 1995). Oviductal secretions contribute to the formation of the oviductal fluid, discussed next.

The oviduct fluid. Reproductive processes occurring in the oviduct are exposed and subjected to regulatory influences of ingredients in the oviductal fluid. Chemical analyses of the oviductal fluid indicated that it is a mixture of constituents derived from the plasma, through selective transudation, plus specific proteins synthesized and secreted by the oviductal epithelium (Leese, 1988). The major classes of components are water, gases (O_2), electrolytes (Ca, Na, K, Cl), non-electrolytes (glucose, fructose, complex carbohydrates) and proteins. Some proteins originate from serum (albumin, immunoglobulins) while others are synthesized de novo in the oviduct [plasminogen activator inhibitor (Kouba et al., 1997) and bovine oviductal glycoprotein (Boyce et al., 1990)]. Functions of oviductal fluid electrolytes and non-electrolytes are reviewed in Leese (1988). De novo synthesized oviductal proteins may affect reproductive

processes such as fertilization, and early embryonic development (Buhi et al., 1997a). More importantly, changes in the optimal milieu of de novo synthesized, secretory oviductal proteins may lead to sub-optimal micro-environments conducive to reproductive failure (Binelli et al., 1999, Chapter 3).

Steroid Regulation and Protein Synthesis

Macromolecules present in oviductal fluid have been suggested to serve important roles in sperm capacitation (Anderson and Killian, 1994), fertilization (Boatman and Magnoni, 1995), and early embryo development (Gandolfi et al., 1989). Therefore, alterations in oviductal biosynthetic activity and protein synthesis and secretion may affect conception rate. Steroid modulation of oviductal synthesis and secretion of proteins has been characterized in sheep (Buhi et al., 1991, Murray, 1993), baboon (Verhage and Fazleabas, 1988) and swine (Buhi et al., 1989; Buhi et al., 1990). Buhi and others (1989) measured the biosynthetic capacity of the oviduct (i.e., rate of incorporation of radiolabeled amino acid precursor into newly synthesized protein) of pigs during the estrous cycle, early pregnancy and in ovariectomized animals following steroid replacement (Buhi et al., 1992). These studies indicated that bioactivity of the oviduct is related to the hormonal status of animals. For example, incorporation rate of radiolabeled leucine was greater when ovariectomized animals were treated with E_2 compared to P_4. These findings were consistent with what was

found with intact animals, where a greater incorporation rate was found in the periestrus stage of the estrous cycle.

It is important to keep in mind that functional regions within the oviduct have specific roles probably associated with particular arrays of secretory products. Thus, it is expected that different steroid environments (e.g., estrous cycle vs. pregnancy) have distinct effects on each oviductal region, characterizing a biosynthetic gradient of proteins across regions. For example, in studies with bulls (Anderson and Killian, 1994), it has been demonstrated that culture medium conditioned by IST tissue at estrus capacitated more sperm than did medium conditioned by AMP. This increase was abolished by heating the conditioned medium and inactivating proteins before incubation with sperm. Staros and Killian (1998) showed that four unidentified oviductal proteins and a P1-like protein (Boice et al., 1990; Binelli et al., 1999; Chapter 3) from non-luteal oviductal fluid would associate with the zona pellucida, suggesting a modulation of sperm/egg binding or embryonic development by oviduct-derived proteins. Biosynthetic protein gradients have been reported in the pig and sheep (Buhi et al., 1992; Buhi et al., 1996; 28, DeSouza and Murray, 1995; Murray, 1993). Moreover, DeSouza and Murray (1995) reported differential secretion of a chitinase-like protein, similar to P1 in response to steroid treatments in sheep, while Buhi et al. (1996) showed differential expression POSP mRNA among oviductal regions in pigs.

While some evidence has accumulated for roles of oviductal secretory proteins on reproductive products, much less is known about roles of embryonic secretory products. To the best of my knowledge, no reports have focused on effects of presence of embryo on the pattern of secretory proteins from the bovine oviduct. Buhi and coworkers (1989), working with porcine oviductal secretory proteins, failed to demonstrate differences in rate of incorporation of non-dialyzable, ^{3}H-leucine labeled molecules between pregnant and cyclic oviducts. Moreover, one-dimensional SDS-PAGE analysis of secretory proteins did not indicate changes in patterns of de novo synthesized, secretory proteins. This indicates that presence of the embryo had little effect on modulating secretion of macromolecules from the oviductal epithelium. In contrast, a recent report (Wakuda et al., 1999) showed that presence of embryos in mice which had their uterotubal junction ligated on day 1 of pregnancy, enhanced implantation rate of embryos transferred to the uterus. This was in comparison with pseudopregnant mice, which had uterotubal junction ligated before or after mating with vasectomized males, and, also mated females which had uterotubal junction ligated before mating (all mice had blastocysts transferred to uterus on day 4). Embryo-dependent factors have not been identified, but clearly influenced embryo development in that species.

Regulation of Reproductive Processes Occurring in the Oviduct

As summarized by Hafez (1993b) and discussed by Harper (1982) and Anderson (1991), transport of unfertilized and fertilized eggs and sperm in the oviduct is regulated by four primary forces: (1) frequency and force of contractions of the oviductal musculature, influenced by endocrine and neural mechanisms; (2) direction and intensity of beating of cilia, which conditions movement of oviductal fluids; (3) secretory activity of non-ciliated cells, which is dependent on the E_2/P_4 ratio; and (4) hydrodynamic properties of luminal fluids. Changes in these factors are modulated by concentrations of ovarian steroids. The outcome of these activities is efficient transport of gametes and embryos and fertilization. Next, I will emphasize the concerted actions of these factors for the mechanisms of egg pick up and fertilization.

Egg pick up. At the time of ovulation, there is a noticeable increase in frequency and amplitude of contractions in the smooth musculature supporting the oviduct. Contractions of the mesotubarium superior and mesosalpinx draw the oviduct in a crescent shape and slide the fimbriae over the surface of the ovary. The fringe-like folds in the INF contract rhythmically to repeatedly touch the ovarian surface (Hafez, 1993a). This pattern of movements constitutes an efficient mechanism to pick up ovulated oocytes. Moreover, maximum density of ciliated cells in the oviduct occur in the INF. During ovulation, the strokes of cilia in the fimbriated portion of the oviduct are synchronized to propel the oocyte

towards the oviductal lumen. Furthermore, volume of oviductal fluid sharply increases 2 days before estrus to reach maximum rate one day after estrus, which coincides with the period of ovulation and reception of oocyte by the INF (Perkins et al., 1965). Muscular contractions, cilia beating and fluid secretion are controlled by ovarian steroids, being stimulated by high periovulatory E_2 concentrations.

Fertilization. In the cow, oocytes are transported rapidly to the site of fertilization, above the isthmoampullar junction, where they spend most of their time in the oviduct (Aref and Hafez, 1973), and then are transported rapidly through the IST into the uterus (Anderson, 1991). A balance among the effects of cilia beating, muscular contractions modulated by catecholamines and fluid flow rate cause this egg "lock up" at the site of fertilization. It has been proposed that the IST of the cow is contracted throughout estrus, and that norepinephrine release after estrus causes relaxation of the IST to allow embryo transit into the uterus (Isla et al., 1989). This is supported by data from El-Banna and Hafez (1970), who showed a dramatic change in the surface area of the IST lumen from estrus to 3 days after estrus (0.06 mm^2 to 1.89 mm^2). Meanwhile, if the animal had been inseminated, sperm are migrating up the reproductive tract and arrive at the IST portion of the oviduct, where their movement is slowed (Hunter and Wilmut, 1982). It is hypothesized that biochemical and biophysical properties of the IST may work to impede upward migration of spermatozoa, including narrow isthmic lumen, viscous isthmic mucous and oviductal

musculature contractions (Ellington, 1991; Hafez 1993b). Within the IST, sperm undergo hyperactivation, which is required for final sperm transport, completion of sperm capacitation and the acrosome reaction. Eventually, spermatozoa become exposed to ampullary fluid, detach from the IST epithelium and continue migration towards the site of fertilization. The control of concerted, opposite direction-movement of sperm and eggs at similar times in the oviduct is intriguing. Perhaps the isthmoampullary junction acts to retain oocytes in the ampulla, while spermatozoa are allowed to enter the IST (Anderson, 1991). Low doses of estrogen cause "tube locking", retaining ova at the isthmoampullary junction, while larger doses promote quick movement through the isthmus and to the uterus (Hawk, 1988).

Oviductal Function and Reproductive Failure in Cattle

The fact that oviductal function is regulated in the multi-factorial, integrated fashion described above could lead one to hypothesize that perturbations in the system could easily lead to reproductive failure. However, in normal cattle, embryonic losses occurring during the time when the embryo is in the oviduct are small, relative to other phases, as described above. This could be interpreted at least in two ways. First, one could say that the oviduct plays only a passive role on the processes of gamete transport, fertilization and early embryonic development. In this view, the oviduct would keep default modes of function (i.e., similar in presence or absence of an embryo), modulated by

patterns of steroids. Gametes and embryos would tolerate mild perturbations in the system and the reproductive processes would be carried out by internal, pre-determined programs, modestly influenced by the oviductal environment. The oviduct would basically provide a physical substratum for events to occur. Leese (1988) suggested that this possibility could be appropriately tested by trying to culture embryos on an epithelium anatomically related to the oviduct, such as the trachea (i.e., ciliated, secretory, containing active chloride ion pump). To support this first possibility, there is the fact that embryos can be matured and fertilized in vitro in the absence oviductal cells, tissue or conditioned medium. Alternatively, one could say that oviduct-gametes/embryos relationships have been optimized in the course of evolution, to become a robust system, with little chance for failure. Specific interactions would be required for success of reproductive processes, including synthesis and secretion of oviductal proteins in a regional and timely fashion. Moreover, such unique set of proteins would interact with gametes/embryos to maximize reproductive output. To test this last possibility, secretory proteins in the oviduct would first need to be identified. Then, removing specific proteins from the system with use of immunoneutralization, knockouts, transgenics and anti-sense models for example, should provide evidence for their importance. For example, addition of specific antibodies for a hamster E_2 -dependent oviduct protein prevents in vitro fertilization (Sakai et al, 1988).

Uterine Function and Reproductive Failure in Cattle

As mentioned before, there is approximately a 30% rate of embryonic losses occurring from days 8 to 16 of early pregnancy, which represents a period of uterine localization of the embryo. In this section I will attempt to describe key aspects of uterine physiology that can be considered when trying to solve the problem of embryonic mortality during this period.

The Uterine Environment

The uterus is considered to be an extension of the oviduct (Bartol, 1999), therefore, several of the principles regarding biophysical and biochemical properties discussed for the oviduct also will apply here. Compared to the oviduct, bovine embryos will spend a much longer interval of time in the uterus (280 days vs 4 days, on average; Catchpole, 1991) which permits a much broader set of communications between the maternal and embryonic units. To support this notion is the fact that although there are not remarkable embryo-induced changes between the cyclic and the pregnant oviduct, this is the opposite for the uterus. The thesis of this section is that presence of the embryo conditions the uterine environment, to support embryonic development.

The uterine cycle. Similar to the oviduct, the uterus also undergoes changes dependent on the stage of the estrous cycle in response to changes in concentrations of ovarian steroids. However, the uterus has the unique role of

controlling length of ovarian cycles (described above) and, as a consequence, its own uterine cycle. The uterine cycle can be divided into a long progestational phase and a short, estrogen-dominated phase (Hansel and Convey, 1983). At the end of progestational phase, the uterus gains the ability to produce and secrete $PGF_{2\alpha}$, which acts to cause structural and functional demise of the CL (McCracken et al., 1971). An immediate consequence of $PGF_{2\alpha}$ actions is a decrease in circulating concentrations of P_4 (Nett et al., 1976). This initiates the estrogen dominated period that lasts until the next ovulation and formation of new CL. Controlling CL life span, the uterus controls the ovarian cycle. During pregnancy, presence of the conceptus blocks luteolytic mechanisms so that the CL remains functional and the uterus remains in a progestational stage until parturition (McCracken et al., 1984). Similar to oviducts, uterine morphology and secretory activity are modulated by ovarian steroids, as discussed below.

Functional anatomy and morphology. The uterus is suspended in the pelvis by the mesometrium, a caudal division of the broad ligament. In cows, the uterus can be described anatomically in two continuous portions, the gestational part of the uterus (consisting of uterine horns and uterine body), and the cervix. Similar to the oviduct, the uterus is a tube-shaped organ, which contains a lumen (Bartol, 1999). Histologically, a cross section of the uterus reveals an inner mucosal layer, the endometrium, an adluminal layer of smooth musculature, the myometrium and an outer, serous peritoneal coat of the uterus, the perimetrium (Bartol, 1999). For the remaining of this discussion, I will focus on characteristics

and processes occurring in the endometrium. The endometrium is lined with a single layer of epithelial cells and contains simple, coiled, tubular glands. Glands are relatively straight at estrus, but become more coiled and complex as progesterone levels rise as the estrous cycle progresses (Hafez, 1993a). Glandular secretions, the hystotroph, constitute a nutrient-rich mixture required for development of the conceptus (Bazer and First, 1983), and will be discussed next. Underneath the luminal epithelium and around the glands is the endometrial stroma, composed of stromal cells distributed in greater or lower density patterns, depending on the location. In the cow, between 100 to 150 aglandular ridges are present, the caruncles (Flood, 1991). During pregnancy, caruncles become attached to specialized areas of the allantochorion of the conceptus, the cotyledons, to form placentomes. Placentomes are units for exchange of gas and nutrients between maternal and embryonic units. A better description on the process of attachment and formation of placentomes is given afterwards in this review. A highly dynamic and organized microvasculature supplies myometrial and endometrial tissues. They originate from uterine branches of the ovarian arteries (supply uterine body and uterine horns), uterine arteries (supply uterine body and uterine horns) and urogenital artery (supplies caudal uterus and cervix). In the cow, uteroovarian relationships exist in that demise of the CL (luteolysis) is regulated by the uterine horn adjacent to the ovary containing CL. Luteolysis is accomplished by countercurrent exchange of the uterine produced luteolysin, $PGF_{2\alpha}$, between the uteroovarian vein and the

ovarian artery. The later is coiled about the surface of the uteroovarian vein (Bartol, 1999). Countercurrent exchange of $PGF_{2\alpha}$ was demonstrated elegantly by Knickerbocker and coworkers (1996). The authors sampled blood originating from uterine branch of ovarian artery(UBOA) and facial artery (FA), and measured changes in $PGF_{2\alpha}$ concentrations in response to a challenge with E_2. There was a greater concentration of $PGF_{2\alpha}$ in UBOA compared to FA, indicating existence of local countercurrent exchange between uterine venous drainage and ovarian artery.

The hystotroph. Hystotroph is the secretions present within the uterine lumen for nourishment of the developing conceptus (Roberts and First, 1983). A broader definition should also include functions such as paracrine regulation of conceptus physiology and development and protection of the conceptus from the maternal immune system. Solymosi and Horn (1994) measured protein content in uterine milk (i.e., hystotroph) of cows and determined that 73% of the dry matter content was composed proteinaceous material. Information on nature of proteins contained in uterine milk is limited in cattle. Electrophoretic analysis reveled at least nine proteins, seven minor and 2 major, which were identified as lactoferrin and acid phosphatase (Bazer and First, 1983). Lactoferrin may have a bacteriostatic function in the uterine luminal environment. The acid phosphatase has basic PI, which is similar to uteroferrin in pigs. Uteroferrin is involved in iron transport to the conceptus (Roberts and Bazer, 1988), but whether bovine acid phosphatase has the same role in cattle is unknown.

MacKenzie and coauthors (1997) reported expression of retinol-binding protein (RBP) in bovine uterus, and monitored steroid modulation of expression of this protein during estrous cycle and early pregnancy. The hypothesized role of RBP is to regulate transport of vitamin A to the conceptus. Finally, expression of growth factors involved in the growth hormone (GH)-insulin-like growth factor (IGF) axis were examined by Kirby and others (1996). They reported expression of IGF-1, IGF-binding protein (IGFBP)-2 and 3 and GH receptor in the uterus. Collectively, uterine milk has functions similar to those of the oviductal fluid, to provide an adequate microenvironment for conceptus development. As the embryo develops, it starts to contribute with its own secretions to the pool of molecules composing the uterine fluid, which becomes more complex and may exert regulatory functions that influence both the conceptus and the uterus.

Regulation of Reproductive Processes Occurring in the Uterus

As mentioned previously, the uterus plays specific roles both during the estrous cycle and during pregnancy. During the estrous cycle, the uterus regulates ovarian function and the ovarian cycle and the uterus in turn is regulated by actions of ovarian steroids. During pregnancy, functions of the uterus include transport, storage and maturation of spermatozoa, recognition and reception of embryos, provision of an embryotrophic environment for conceptus development during gestation, and expulsion of fetus and placenta at parturition (Bartol, 1999). At this physiological state, uterine function is regulated

both by ovarian steroids and embryonic bioactive molecules. During pregnancy, misregulation of uterine function may lead to embryonic mortality. For the remaining of this section I will focus on the mechanisms regulating $PGF_{2\alpha}$ production from the cyclic uterus, and on mechanisms of maternal recognition of pregnancy related to CL maintenance occurring in the pregnant uterus.

Cyclic uterus. The reason for the uterine cycle is to provide repeated opportunities for pregnancy at relatively short intervals. In practice, the turning point in the uterine cycle is the commitment to either luteolysis or pregnancy. In the absence of pregnancy, the progestational stage is finished by the uterus-induced demise of the P_4 source. Demise of the CL is accomplished by pulsatile secretion of uterine $PGF_{2\alpha}$ (Nancarrow et al., 1973; Kindahl et al., 1976). Then, the estrogen-dominated uterus prepares for reception of sperm, initially, and reception of the early developing embryo. This cycle repeats until successful establishment of pregnancy. However, early pregnancies will be terminated if the uterine cycle is not interrupted.

Secretion of $PGF_{2\alpha}$. During the estrous cycle, the presence of two distinct patterns of $PGF_{2\alpha}$ release is easily distinguishable : a basal release and a stimulated, pulsatile release. Initial measurements of $PGF_{2\alpha}$ were performed in samples collected from the venous drainage of the uterus, which required surgical cannulation (Nancarrow et al., 1973). Measuring peripheral concentrations of 15-keto-13, 14-dihidroprostaglandin $F_{2\alpha}$, (PGFM) the main metabolite of $PGF_{2\alpha}$ found in the circulation, facilitated study of such patterns

(Kindahl et al., 1976). It was determined that for most of the estrous cycle, basal secretion of PGFM ranged from 25 to 70 pg/ml in one heifer and from 60-100 pg/ml in a second heifer. However, around the time of luteolysis, four peaks of about 500 pg/ml and four peaks of about 250 pg/ml were observed for the first and second heifers, respectively (Kindahl et al., 1976). Despite the clear among animal variability in this small experiment, there was an evident decrease in P_4 concentrations, from ~5 ng/ml to less than 1 ng/ml, within 24 hours after the first PGFM peak for both heifers.

Pulsatile release of $PGF_{2\alpha}$. Generation of $PGF_{2\alpha}$ pulses requires presence of a stimulatory signal and a responsive uterus. A responsive uterus contains receptors for the stimulatory signal, functional intracellular pathways to transduce the stimulus into a secretory pulse and adequate amounts of substrate for $PGF_{2\alpha}$ synthesis.

In cattle, nature of the stimulatory signal for production of luteolytic pulses of $PGF_{2\alpha}$ remains unclear. It has been accepted generally that oxytocin is the major stimulator of $PGF_{2\alpha}$ secretion in cattle. Armstrong and Hansel (1959) demonstrated that exogenous oxytocin caused luteolysis in heifers. Moreover, injections of oxytocin increased concentrations of $PGF_{2\alpha}$ in the uterine vein (Milvae and Hansel, 1980) and increased concentrations of PGFM in peripheral circulation (Lafrance and Goff, 1985) in cows. However, while oxytocin is able to stimulate $PGF_{2\alpha}$ secretion in these experiments, it remains unclear whether oxytocin is in fact required for the process of luteolysis. In a recent report,

Kotwica and others (1997) demonstrated that administration of an efficacious oxytocin receptor antagonist (CAP-527) failed to block normal luteolysis in cows. Moreover, treatment of endometrial explants from days 16-17 cyclic cows with oxytocin failed to stimulate secretion of $PGF_{2\alpha}$, both in static (Arnold et al., 1999) and in perifusion (Del Vecchio et al., 1990) culture systems. This raises the possibility that perhaps ligands other than oxytocin are required for luteolysis in cattle. Alternatively to oxytocin, possible stimulators of pulsatile $PGF_{2\alpha}$ secretion include E_2 and LH.

Irradiation of ovarian follicles, which reversibly eliminates production of follicular E_2, delays luteolysis and extends the length of the estrous cycle (Hughes et al., 1987). Thus, follicular E_2 plays a major role in these events. In heifers, Thatcher and coworkers (1986) demonstrated that injections of E_2 on day 13 of the estrous cycle stimulated release of PGFM starting 3 hours after the injection, peaking at 6 hours and returning to basal levels by 10 hours. In addition, heifers injected with E_2 underwent luteolysis 96 hours after injections, while it took 125 hours for control heifers. To confirm that the PGFM increases measured in the peripheral circulation represent $PGF_{2\alpha}$ of uterine origin, Knickerbocker and others (1986) measured a sharp increase in $PGF_{2\alpha}$ in the uterine vein of cows treated with E_2. Collectively, these findings indicate that both endogenous and exogenous E_2 are able to stimulate secretion of $PGF_{2\alpha}$ and to cause luteolysis.

A novel concept regarding control of luteolysis involves the actions of LH in the endometrium. Friedman et al. (1995) reported the presence of LH binding sites in bovine endometrium that were maximal in endometrium from days 15 to 17 which corresponds to the time of luteolysis. In addition, production of $PGF_{2\alpha}$ was stimulated when endometrial cells from days 15 to17 of the estrous cycle were treated with LH in vitro. In a series of preliminary experiments (Fields, personal communication) ovariectomized, P_4-treated cows were injected with either saline or E_2 and 4 hours later injected with either saline or human chorionic gonadotropin (hCG, a long half life LH analog). Concentrations of PGFM in plasma were elevated only for groups pre-treated with E_2. In addition, hCG injection elicited a pronounced release of PGFM compared to saline. It can be concluded that exposure to E_2 is required for the endometrium to secrete PGFM in response to LH. Physiologically, it could be hypothesized that E_2 acts at the endometrium to enhance responsiveness to circulating LH, thereby evoking $PGF_{2\alpha}$ secretion during luteolysis. Indeed, a decline in P_4 will elevate plasma LH that may contribute to a continued secretion of $PGF_{2\alpha}$ to re-enforce the luteolytic process. Mechanistically, this could be accomplished by increasing concentration of LH receptors in the endometrium or by connecting intracellular pathways stimulated by LH with the $PGF_{2\alpha}$ secretory machinery.

In summary, it is doubtful that oxytocin is the sole stimulator of pulsatile secretion of $PGF_{2\alpha}$. It is more probable that other effectors such as E_2 and LH act in concert with oxytocin to stimulate luteolysis.

Since the dominant paradigm in the field of luteolysis has been that oxytocin is the major stimulator of $PGF_{2\alpha}$ pulsatile secretion, experiments to test presence of a responsive uterus had as an endpoint development of oxytocin receptors and secretion of $PGF_{2\alpha}$ in response to oxytocin. Based on this paradigm, it has been well established that exposure of the uterus to progesterone is required for the uterus to acquire responsiveness to oxytocin. McCracken (1980) proposed that P_4 has the ability to inhibit synthesis of E_2 receptors, and synthesis of oxytocin receptors is an E_2 -dependent process. As long as P_4 inhibits synthesis of E_2 receptors, E_2 is unable to stimulate synthesis of oxytocin receptors. Moreover, McCracken and others (1984) suggested that the uterus eventually becomes refractory to inhibitory effects of P_4, allowing oxytocin receptors to be expressed, which leads to pulses of $PGF_{2\alpha}$. Lafrance and Goff (1988) treated long-term ovariectomized heifers with P_4 for 0, 7, 14 or 21 days then measured PGFM in response to an oxytocin challenge. After 7, 14 or 21 days of P_4 -priming there was a significant increase in plasma PGFM after oxytocin injection, but no increase was noticed in animals that did not receive P_4. Silvia and coauthors (1991) put forth the question of whether requirement for long term exposure to P_4 is due to stimulatory effects that take at least 10 days to build up (7 days in Lafrance and Goff, 1988) or to slow development of the condition whereby the uterus is desensitized to P_4 inhibitory effects. Collectively, the concept of a responsive uterus means that this organ has been primed by P_4, and as a result, the uterus becomes responsive to the luteolytic stimulus.

I will next review intracellular pathways for generation of the pulsatile secretion of $PGF_{2\alpha}$. Little experimentation has been done to uncover potential intracellular pathways activated by estradiol or LH, so focus is on the well studied and established pathway of oxytocin stimulation (Flint et al., 1986; Burns et al., 1997; Thatcher et al., 1997). Oxytocin receptors start to increase in the P_4 - primed, responsive uterus. Oxytocin originating from the neurohypophyseal lobe of the pituitary gland binds to the seven transmembrane-domain, G protein-coupled receptors and activates phospholipase C (PLC). The PLC cleaves membrane phosphotydilinositol bisphosphate, yielding inositol trisphosphate (IP_3) and diacylglycerol (DAG). The IP_3 binds to specific receptors in the endoplasmic reticulum resulting in release of calcium from internal stores into the cytosolic compartment. The DAG activates protein kinase C (PKC), leading to serine phosphorylation of cytosolic, calcium-dependent phospholipase A_2 (PLA_2), probably through a MAP-kinase dependent pathway (Lin et al., 1993). The IP3-stimulated increase in cytosolic calcium acts to further stimulate PLA_2 activity (Clark et al., 1991). Stimulated PLA_2 translocates to the membrane where phospholipid substrates are located (Clark et al., 1991). Activated, membrane-bound PLA_2 cleaves arachidonic acid (AA) from phospholipids. Free AA is converted to prostaglandin H_2 (PGH_2) by the enzyme cyclooxygenase-2 (COX-2). Prostaglandin $F_{2\alpha}$ synthase converts PGH_2 into $PGF_{2\alpha}$, which is then released into the uterine circulation. In the endometrium, this process occurs preferentially in epithelial cells compared to stromal cells (Danet-Desnoyers et

al., 1994). As discussed beforehand, $PGF_{2\alpha}$ gains access to the ovary through a counter-current mechanism. Binding of $PGF_{2\alpha}$ to receptors in the CL stimulates release of luteal oxytocin that in turn binds oxytocin receptors in the endometrium to elicit further release of $PGF_{2\alpha}$, characterizing a positive feedback loop.

Arnold and coauthors (1999) demonstrated that a responsive uterus is not necessarily a oxytocin-responsive uterus. They incubated endometrial explants obtained from day 17 cyclic cows with oxytocin or with intra-cellular stimulators of the $PGF_{2\alpha}$-generating cascade described above. They showed that despite oxytocin failure to stimulate $PGF_{2\alpha}$ secretion, the stimulator of PKC activity, phorbol 12, 13 dibutyrate (PDBu), and the stimulators of PLA_2 activity, calcium ionophore and melittin, were able to induce $PGF_{2\alpha}$ release acutely. This supports the notion that alternative ligands to oxytocin can play a role on pulsatile secretion of $PGF_{2\alpha}$.

A final comment on the role of P_4 -priming of the uterus for pulsatile $PGF_{2\alpha}$ secretion, relates to P_4 ability to induce accumulation of lipid droplets in bovine uterine epithelial cells (Brinsfield and Hawk, 1973). In mice, such lipid droplets contain phospholipids (Silvia et al., 1991), which are substrate for PLA_2 and source of AA, as mentioned above. Progesterone also induces synthesis of COX-2 (Raw et al., 1988).

Pregnant uterus. In cattle, the vast majority of embryos are found in the uterine horn ipsilateral to the ovary where ovulation occurred, indicating a readily

"attachable" embryo, which only migrates minimally within the uterus (Flood, 1991). Embryos undergo rapid morphological changes in the first 3 weeks of pregnancy. After a series of cellular divisions, formation and hatching of blastocysts (day 9-10 after ovulation; Betteridge and Flechon, 1988), conceptuses start to elongate on day 12 (Betteridge et al., 1980), to occupy the whole length of the uterine horn ipsilateral to the CL by day 17 and to reach the tip of the contralateral horn on day 21 (Kastelic et al., 1988; Flood, 1991). The first intimate connection between the conceptus and the uterus occurs between days 18 and 20 of pregnancy, when numerous papillae penetrate the openings of uterine glands (Guillomot et al., 1981). There is intimate contact of maternal and embryonic tissues starting with apposition of apical cell membranes of aligned epithelia from both units. Actual adhesion begins around day 22 and is completed on day 27 after insemination. Adhesion is characterized by interdigitation of embryonic and maternal microvilli (Flood, 1991). The next series of events include development of placentomes and growth of placental tissues.

Maternal recognition of pregnancy associated with CL maintenance

As mentioned previously, the turning point in the uterine cycle is the commitment to either luteolysis or pregnancy. In cattle, commitment to pregnancy is only accomplished if adequate signaling exists between maternal and embryonic units. Maternal recognition of pregnancy has been defined as the process by which the periattachment conceptus signals its presence to the

maternal unit, as reflected by maintenance of the CL (Short, 1969; reviewed in Hansen, 1991). More specifically, the process of maternal recognition of pregnancy requires that embryonic molecules interact with the uterine endometrium and change its program, so that pulsatile secretion of $PGF_{2\alpha}$ is blocked and thereby luteolysis is impeded. The net result is continuous secretion of P_4 by the CL, which is required for continuation of pregnancy. Roles of P_4 include continuous stimulation of uterine secretions and inhibition of smooth muscle contractions (Hafez, 1993a). In cattle, the critical period for maintenance of pregnancy is around day 17 of the estrous cycle. Betteridge and others (1980) transferred embryos to synchronized recipients and demonstrated that pregnancy was only maintained if embryos were transferred prior to day 17. Moreover, inter-estrus interval increased from 20 to 25 days when conceptuses were removed on day 17 vs. day 15 of pregnancy (Northey and French, 1980).

Based on the model proposed above, pregnancy effects on suppression of pulsatile $PGF_{2\alpha}$ could be exerted at several levels: (1) suppression of the $PGF_{2\alpha}$-releasing stimulus (i.e., oxytocin, LH, E_2), (2) alterations of the P_4-primed uterus (i.e., $PGF_{2\alpha}$-synthesizing machinery), and (3) decrease in substrate required for $PGF_{2\alpha}$ synthesis (i.e., AA). Another possibility is presence of an conceptus-induced luteoprotective action, where CL would become less susceptible to luteolytic effects of $PGF_{2\alpha}$. However, since $PGF_{2\alpha}$ pulses are effectively blocked during early pregnancy, this possibility will not be considered in this discussion.

There is good evidence for pregnancy-induced suppression of luteolytic stimulus in cattle relative to the attenuation of E_2 effects. Pregnant cows have reduced circulating concentrations of E_2 (Pritchard et al., 1994), probably as a result of reduced folliculogenesis (total production of follicles) and decreased production of E_2 per follicle (decreased aromatase activity; Thatcher et al., 1991). Moreover, in day 18 pregnant cows, administration of E_2 stimulates only a modest increase in $PGF_{2\alpha}$ secretion, indicating that presence of the conceptus attenuates E_2 effects (Thatcher et al., 1984).

Regarding alterations on the P_4-primed uterus, Arnold and others (1999) incubated endometrial explants obtained from day 17 cyclic or day 17 pregnant cows with intracellular stimulators of $PGF_{2\alpha}$ synthesis, and measured concentrations of $PGF_{2\alpha}$ secreted into the culture medium. Melittin, PDBu and calcium ionophore each stimulated release of $PGF_{2\alpha}$ from explants of cyclic cows compared to control treatment (medium alone). In contrast, all stimulators mentioned above failed to induce release of $PGF_{2\alpha}$ in explants originated from pregnant cows. This indicated that pregnancy affected the intracellular $PGF_{2\alpha}$-generating machinery to suppress its ability to stimulate $PGF_{2\alpha}$. Interpretation of these data suggests that pregnancy may have inhibitory effects at each of the steps stimulated by treatments, which include PKC (PDBu) and PLA_2 (melittin, ionophore). Alternatively, pregnancy may affect a distal, convergence point in the pathway, for example, at the COX-2 level. Effects on the enzymatic machinery can be to decrease expression and/or activity of PKC, PLA_2 and COX-

2. In fact, existence of a pregnancy-induced inhibitor of COX-2 activity has been found in the endometrium of cows (Basu and Kindahl, 1987; Gross et al, 1988). Danet-Desnoyers and others (1993) identified linoleic acid as the active molecule in bovine endometrium which acted to decrease COX-2 activity. Moreover, linoleic acid acted as a competitive inhibitor of AA on a $PGF_{2\alpha}$ generator assay (Thatcher et al. 1994b). It is possible that altered lipid metabolism in the pregnant endometrium increases availability of linoleic acid to inhibit COX-2 activity and thereby decrease $PGF_{2\alpha}$ production.

Finally, pregnancy could change lipid composition and metabolism in the endometrium to inhibit $PGF_{2\alpha}$ synthesis. Thatcher and others (1995) compared concentrations of free linoleic and free AA in endometrial microsomes from day 17 cyclic and pregnant cows. They found that pregnancy decreased concentrations of AA and increased concentrations of linoleic acid compared to estrous cycle, to result in a change of the ratio of linoleic to AA of 0.6 to 2.4 in endometrium between cyclic and pregnant cows.

Effectors of maternal recognition of pregnancy: IFN-τ

A considerable amount of research focused on identification and purification of conceptus products with the $PGF_{2\alpha}$-secretion inhibitory activity required for maintenance of pregnancy. A family of molecules has been identified as the embryonic antiluteolytic factor in ruminants, named IFN-τ (Thatcher, 1999). For a historical prospective, see Martal et al. (1979), Godkin et

al. (1982), Bartol et al. (1985), Helmer et al. (1987), Imakawa et al. (1989) and Roberts et al. (1992). Isoforms of bIFN-τ are glycosylated, have molecular weights between 22 and 24 kD and vary in isoelectric forms between PI 6.3 and 6.8 (Helmer et al., 1987; Anthony et al., 1988).

Antiluteolytic effects of bovine bIFN-τ (bIFN-τ) have been examined both in vivo and in vitro. Intrauterine infusions of highly enriched bIFN-τ complex (Helmer et al., 1989b) and recombinant bIFN-τ (Meyer et al., 1995) extended lifespan of CL in cows, compared to control infusions. Moreover, $PGF_{2\alpha}$ release in response to an oxytocin injection was suppressed in day 17 cyclic cows infused with recombinant bIFN-τ compared to controls (Meyer et al., 1995).

Danet-Denoyers and others (1994) tested the ability of bIFN-τ to suppress basal and oxytocin-stimulated secretion of $PGF_{2\alpha}$ from primary cultures of endometrial epithelial cells obtained from day 15 cyclic cows (Danet-Desnoyers et al., 1994). Twenty four hour-incubation with bIFN-τ reduced both basal and oxytocin-stimulated secretion of $PGF_{2\alpha}$. This agrees with data from Meyer and coworkers (1996) and Xiao et al. (1999). Meyer and others (1996) reported that endometrial epithelial cells obtained from cows which received intrauterine infusions of bIFN-τ secreted less basal and oxytocin-stimulated $PGF_{2\alpha}$ compared with cows infused with a control protein. Moreover, Xiao and coworkers (1999) cultured endometrial epithelial cells obtained from cows of days 1 to 4 of the estrous cycle in presence of oxytocin or a combination of oxytocin and bIFN-τ.

Similar to the data described above, bIFN-τ effectively reduced both oxytocin- and phorbol ester-stimulated $PGF_{2\alpha}$ secretion. In contrast, Asselin and others (1998) showed that bIFN-τ increased secretion of $PGF_{2\alpha}$ from endometrial epithelial cells from days 1 to 5 of the estrous cycle. However this effect was only significant when extremely high doses of bIFN-τ (20 μg/ml) were used. Collectively, these data support the concept that bIFN-τ interacts with endometrial epithelium and affects the $PGF_{2\alpha}$-generating machinery to decrease $PGF_{2\alpha}$ production. To further test this possibility, Arnold and others (1999) infused either bIFN-τ or a control protein (bovine serum albumin) in the uterus of cows from days 14 to 17 of the estrous cycle. Secretion of $PGF_{2\alpha}$ was measured in medium conditioned by endometrial explants cultured in presence of specific intracellular stimulators of $PGF_{2\alpha}$ synthesis. Incubations with calcium ionophore and PDBu stimulated $PGF_{2\alpha}$ secretion compared to medium alone in endometrium from control cows, but not from bIFN-τ -treated cows. In contrast, melittin stimulated secretion of $PGF_{2\alpha}$ from explants originating from bIFN-τ -infused cows. Overall, their data indicated that in vivo treatment with bIFN-τ attenuated $PGF_{2\alpha}$ production probably at the level of PKC, since PDBu stimulation of $PGF_{2\alpha}$ was reduced by bIFN-τ, whereas melittin stimulated $PGF_{2\alpha}$ secretion. This is in variance with the ubiquitous inhibitory effects of pregnancy on stimulated $PGF_{2\alpha}$ secretion (mentioned above), suggesting that other products

of pregnancy, and not bIFN-τ alone, probably also operate to inhibit $PGF_{2\alpha}$ production.

In an effort to pinpoint specific enzymes that bIFN-τ altered in the $PGF_{2\alpha}$-generating cascade, Xiao and others (1999) measured messenger ribonucleic acid (mRNA) and protein expression for COX-2 in endometrial epithelial cells treated with oxytocin and with oxytocin in combination with bIFN-τ. Oxytocin maximally stimulated COX-2 mRNA and protein from 3 to 24 hours compared to controls. Treatment with bIFN-τ reduced this effect of oxytocin, and this was consistent with a reduction in $PGF_{2\alpha}$ secretion in medium. In contrast, Asselin and coworkers (Asselin et al., 1997) found that bIFN-τ actually stimulated expression of COX-2, which would contradict the antiluteolytic role of bIFN-τ. However, they also reported that bIFN-τ stimulated expression of an endometrial prostaglandin E_2-9-ketoreductase, which catalyzes the conversion of $PGF_{2\alpha}$ into PGE_2 (Asselin and Fortier, 1998). Since PGE_2 has been shown to have luteo-protective actions (Pratt et al., 1977), they proposed a model whereby bIFN-τ actually stimulates the $PGF_{2\alpha}$-generating machinery, but a conversion of $PGF_{2\alpha}$ to PGE_2 at the end of the cascade would support an antiluteolytic effect of bIFN-τ.

It is expected that in order to stimulate intracellular changes resulting in decreased $PGF_{2\alpha}$ production, bIFN-τ needs to stimulate a receptor-mediated mechanism of signal transduction. Such a mechanism should evoke intracellular

second messengers to ultimately regulate molecules involved in the generation of $PGF_{2\alpha}$. Such regulation could involve synthesis of proteins inhibitory to the $PGF_{2\alpha}$ production cycle, or, could acutely activate molecules already present in the cell to suppress $PGF_{2\alpha}$ stimulatory actions. There is limited information on the nature of IFN-τ receptors. Knickerbocker and Niswender (1989) measured numbers of unoccupied binding sites for IFN-τ in endometrium of cyclic and pregnant sheep. Number of unoccupied binding sites decreased for both cyclic and pregnant ewes from day 4 to day 12. Then it increased for cyclic animals, but was still decreased for pregnant ewes, indicating that bIFN-τ binding sites were possibly being occupied by conceptus-secreted IFN-τ. Interestingly, affinity for binding sites increased after day 12 for pregnant ewes but decreased for cyclic ewes.

Hansen and coauthors (1989) reported use of cross-linking experiments to characterize association of iodinated ovine IFN-τ to membrane peptides. They identified binding of IFN-τ to both 100 and 70 kD membrane polypeptides. Comparison of binding kinetics of IFN-τ with IFN-α in this experiment suggested existence of different receptors for these two ligands. However, Li and Roberts (1994) showed a reciprocal displacement of IFN-τ and IFN-α from bovine endometrial cell membranes, suggesting that binding sites for these two molecules were the same. Recently, Han and Roberts (1998) reported cloning and characterization of receptors for IFN-τ in cattle endometrium. Sequences of

receptor subunits IFNαR1 and IFNαR2 are similar to ones utilized by other type I interferons such as IFN-α. However, these receptors were not linked with functional data, to demonstrate that such subunits are necessary and sufficient to suppress $PGF_{2\alpha}$ synthesis.

The JAK-STAT Pathway

The observations above lead to the assumption that bIFN-τ stimulated a signal transduction system, the JAK-STAT pathway, similar to other type I interferons (Schindler et al., 1992; Darnell et al., 1994; Darnell, 1997; Figure 2-1). In this paradigm, interferon receptors do not contain intrinsic kinase activity, but they are physically associated with protein tyrosine kinases from the Janus family (JAK kinases). Binding of interferon to its receptor causes phosphorylation of tyrosine residues in the JAK kinases and in the cytoplasmic tail of the receptor. The tyrosine phosphorylated receptor attracts signal transducer and activation of transcription, or STAT, proteins to close contact. Members of the STAT family of proteins then become phosphorylated on tyrosine residues and form homo- and hetero-dimers. Dimerized STATs migrate to the nucleus where they bind to the specific regulatory elements located in the promoter region of interferon-regulated genes. In this manner, STAT proteins

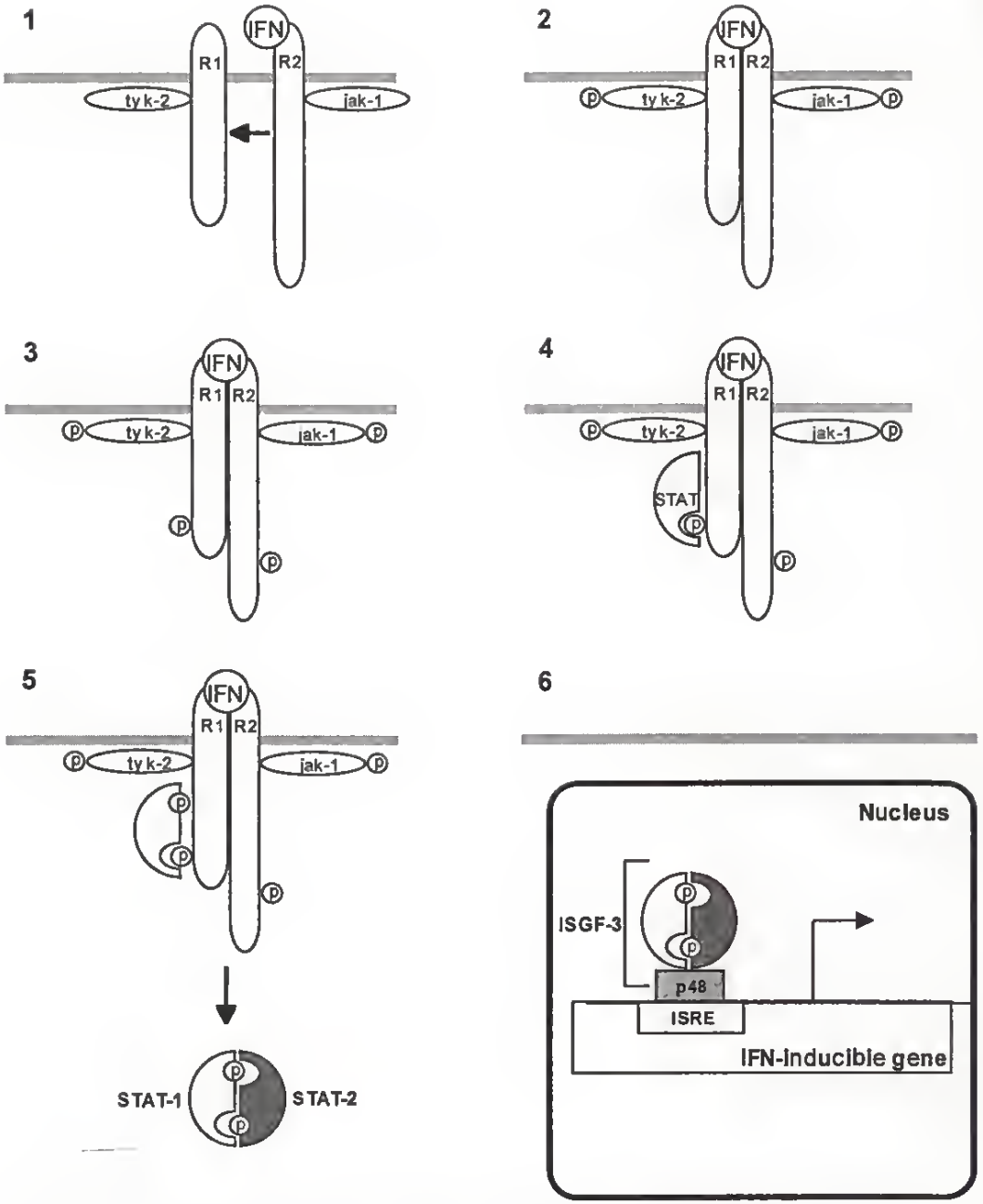


Figure 2-1. The JAK-STAT pathway of signal transduction and gene activation.

1) Binding of type I interferon (IFN) to the interferon-α-receptor (R)-2 chain causes recruitment of R1; 2) dimerization of IFN receptor complex causes reciprocal tyrosine phosphorylation of associated JAK kinases (tyk-2 and jak-1); 3) JAK kinases phosphorylate receptor subunits in tyrosine residues; 4) unphosphorylated, cytosolic STAT proteins bind receptor complex through SH2 domains present in STAT proteins; 5) JAK kinases phosphorylate tyrosine residues in bound STATs, STATs dissociate from receptor complex and associate in a heterodimer (STATs 1 and 2); 6) dimerized STATs translocate to the nucleus, bind to the DNA binding protein p48, forming the ISGF-3 transcription complex, which stimulate synthesis of IFN-inducible genes.

stimulate transcription of genes and synthesis of interferon-specific proteins. I will next examine characteristics of molecules involved in this pathway, and then describe evidence for existence of this pathway in the bovine endometrium.

Type I interferon receptors

Type I interferon receptor consists of two chains, IFNαR1 and IFNαR2, which can be presented in different forms. The IFNαR1 is present as a full chain (IFNαR1a) and as a shorter splice variant (IFNαR1s). The IFNαR2 chain exists in soluble, short and long forms, designated IFNαR2a, IFNαR2b and IFNαR2c respectively. Probably IFNαR1a and IFNαR2c are the predominant forms (Petska, 1997). Petska (1997) reviewed a series of experiments where the different IFNαR chains were expressed in Chinese hamster ovary cells, and ability of different type I interferons to signal through the different chain combinations was evaluated. There is a remarkable diversity of such interactions, in which specific interferons can only signal through specific combinations of chains, but not others. Petska (1997) proposes that differential expression of individual chains and ability of individual interferons to signal through specific chain arrangements confers tissue-specific responsiveness to interferons. For example, Platanias and coworkers (1996a) reported that IFN-β signaling requires association of IFNαR1 with p100, a tyrosil phosphoprotein, which was later identified as a particular chain of the interferon receptor complex. To the best of my knowledge, these types of experiments have not been

conducted in bovine reproductive tissue, to test signaling ability of bIFN-τ. Instead of the antiviral assays used in the experiments mentioned above (Pestka, 1997), functional assays measuring suppression in synthesis of $PGF_{2\alpha}$ from endometrial cells would be in order. Moreover, existence of a bIFN-τ-specific receptor chain remains elusive.

Colamonici and coworkers (1994a; 1994b) demonstrated that the tyrosine kinase $p135^{tyk2}$, or tyk-2 is associated physically with the IFNαR1 chain of the interferon receptor. Immunoblots revealed the ability of monoclonal antibodies to IFNαR1 and to tyk-2 to reciprocally coimmunoprecipitate both proteins. Association of tyk-2 was mapped to a 46-amino acid juxtamembrane region of the IFNαR1 chain. Furthermore, they demonstrated that tyk-2 could directly phosphorylate tyrosine residues in the IFNαR1 chain after stimulation with IFN-α (Colamonici et al., 1994b). Besides binding to extracellular interferons and associating with JAK kinases, the interferon receptor complex also has other functions in the JAK-STAT pathway. The unstimulated IFNαR2 chain may contain associated unphosphorylated STAT proteins (Li et al., 1997). Binding of interferon brings IFNαR2 and IFNαR1, which contains tyk-2, together. Dimerization of receptor chains elicits transfer of STATs to the IFNαR1, where STATs become tyrosine phosphorylated. This confirms the previous finding that tyrosine 466 in the chain of IFNαR1 acts as a docking site for association of the SH2 domain of STAT-2, and such binding is required for tyrosine

phosphorylation of STAT-2 by tyk-2 (Yan et al., 1996). Similar to STAT-2, STAT-3 activation also requires binding to IFNαR1 (Yang et al., 1996). Furthermore, it has been demonstrated that phosphotyrosine modules (i.e., sequence of amino acids surrounding the tyrosine residue in the receptor chain) play a major role in selecting which STAT binds (Gerhartz et al., 1996). The authors demonstrated that a two point mutation in the phosphotyrosine module changed the specificity of interferon-gamma receptor from STAT-1 to STAT-3.

Chains of the interferon receptor may also play roles independent of the JAK-STAT pathway. For example, Abramovich and others (1997) reported binding of a protein-arginine methyltransferase to the IFNαR1 chain. This finding suggests that methylation of proteins may be a signaling mechanism complementary to tyrosine phosphorylation, and methylation may be required for full stimulation by interferons. In fact, cells deficient in this methylase activity by antisense become less sensitive to the antiproliferative effect of interferons. Finally, Platanias and coauthors (1996b) reported that the interferon receptor mediates tyrosine phosphorylation of insulin receptor substrate 2 (IRS-2). The IRS-2 molecules associate with IFNαR1 and become phosphorylated by tyk-2. Moreover, phosphorylated IRS-2 associates with the p85 regulatory subunit of the phosphatydilinositol 3'-kinase, suggesting that this kinase participates in the interferon signaling cascade downstream from IRS-2. Collectively, the examples presented above illustrate actions of the multifunctional interferon receptor. It is

tempting to speculate that some of such actions may be required for the antiluteolytic roles of bIFN-τ in the endometrium.

JAK kinases

Janus kinases or JAKs tyk-2 and jak-1 are associated respectively with IFNαR1 and IFNαR2 and are involved in tyrosine phosphorylation of STAT proteins. The carboxy-terminal domains of the jak kinases share considerable sequence homology with the catalytic domains of other protein tyrosine kinases. The amino-terminal half of the jaks contains regions of sequence homology to other members of the jak family and the extreme amino-terminal domain probably is involved in association with interferon receptor chains (Williams and Haque, 1997). Ligand-mediated dimerization of interferon receptor chains is required for interferon-stimulated signal transduction. Dimerization evokes reciprocal tyrosine phosphorylation and consequent activation of JAKs associated with interferon receptor chains (Ihle et al., 1995). Phosphorylation of the kinase is the first of three tyrosine phosphorylations culminating in STAT activation. Activated JAKs phosphorylate tyrosine residues on the interferon receptor chains, which serve as docking sites for STATs, as mentioned above. Lastly, STATs are phosphorylated by the JAKs (Darnell, 1997). Activated JAKs are not specific for particular STATs. Different receptors can activate the same STATs through different JAKs. Moreover, STAT docking sites can be interchanged between different cytokine receptors, and the STAT specific for the

docking site present will be activated by binding of the ligand specific for the extracellular domain of the receptor (Stahl et al., 1995). Therefore, STAT activation is determined more by specific interactions between STATs and their receptors than by specific JAKs associated with receptor chains.

STATs

Unlike other common intracellular second messengers, STATs not only convey the extracellular signal to the interior of the cell, but they themselves carry such a signal to the nucleus, acting as transcription factors to activate transcription of genes induced by particular ligands. I will focus this discussion on STATs 1, 2 and 3, although STATs 4 to 6 have been described (Darnell, 1997). STAT-1α and 1β are encoded by alternative splicing of a single mRNA transcript. Human STAT-1α consists of 750 amino acids, while the extreme carboxy-terminal 38 amino acids are missing for STAT-1β. STAT-2 is composed of 851 amino acids. STATs 1, 2 and 3 have significant sequence homology (Fu et al., 1992; Zhong et al., 1994). The domain distribution in the STAT molecule includes a centrally-located DNA-binding domain, a carboxy-terminal transcription activation domain, and SH2 and SH3 domains located in between them (Fu, 1992; Figure 2-2). The SH2 domain allows docking to tyrosine phosphorylated sites in the IFN and cytokine receptors, as discussed above, and also STAT dimerization. SH2 domain sequences are specific for each STAT, but mutant STATs 1 and 3, in which SH2 domains were swapped, completely

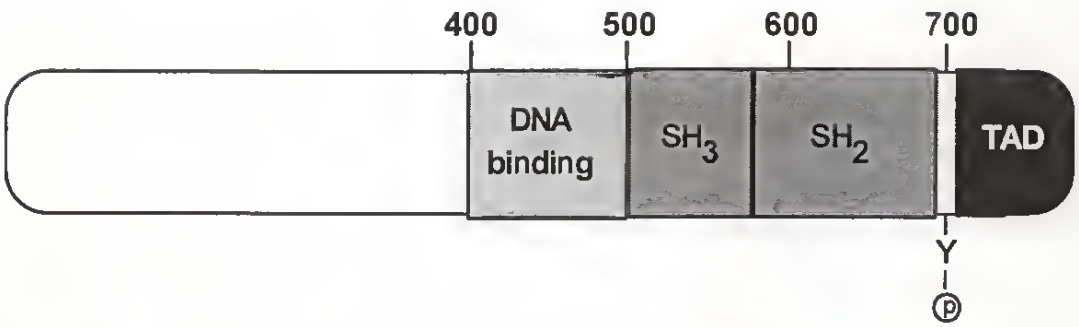


Figure 2-2. Domain structure of the STAT-1 protein. The diagram represents the linear structure of STAT-1 oriented in a amino- (leftmost) to carboxy- terminus sequence. DNA binding domain, SRC homology 2 (SH2) domain, SH3 domain and transcription activation domain (TAD) are represented in the sequence they occur in the STAT-1 molecule. The site of tyrosine (Y) phosphorylation is also represented.

reversed their specificity for interaction with specific phosphotyrosine motifs (Hemmann et al., 1996). This indicates that the SH2 domain is the sole determinant of specific STAT factor recruitment to receptors. STATs contain a unique tyrosine residue in the carboxy-terminal region (Y701, Y690 and Y705 for STATs 1, 2 and 3 respectively). A recently developed model for STAT activation (Li et al., 1997; Figure 2-1) proposes that unphosphorylated STATs 1 and 2 are associated with the IFNαR2 chain. Binding of interferon causes dimerization of this chain with IFNαR1. Tyrosine phosphorylated residue 466 of IFNαR1 binds the SH2 domain of STAT-2, which is then phosphorylated on tyrosine 690, providing a docking site for the SH2 domain in STAT-1. STAT-1 is phosphorylated on tyrosine 701, and then dimerizes with STAT-2 through reciprocal binding of tyrosine phosphorylated residues with SH2 domains. However, an unsolved question is what drives SH2 domains of STATs to dissociate from a higher affinity interaction with receptor phosphotyrosine to form

dimers which association is mediated by a lower-affinity phosphotyrosil interaction (Greenlund et al., 1995). In light of this question, Gupta and coauthors (1996) proposed an alternative model for STAT binding and dimer formation. After binding to the receptor phosphotyrosine motif, the STAT shifts its target to the tyrosine motif in the tyrosine kinase. Tyrosine phosphorylation of STAT would cause a conformational change to destabilize this interaction with the kinase, and STATs would then be driven to form more energy-stable interactions with other STATs and form dimers. They based this model on the finding that SH2 domains from STATs 1 and 2 bind with high affinity to phosphotyrosine motifs on JAK kinases.

STAT dimers are competent to bind DNA. Known DNA binding heterodimers are STAT 1:2 and STAT 1:3 (strong binding) and STAT 2:3 (weak binding). Homodimers are STAT 1:1 and STAT 3:3 (strong) and STAT 2:2 (form seldom in absence of STAT-1; Darnell, 1997). In variance with the notion that tyrosine phosphorylation is required for STAT dimerization, Stancato and coworkers (1996) demonstrated that STAT complexes exist in the cytosol of unstimulated cells. Moreover, such association was independent of tyrosine phosphorylation, since the Y701F STAT-1 mutant still bound to STAT-2 in reticulocyte lysates. Such an interaction was weak, since it was not observed in extracts obtained with high-salt, detergent-containing buffers.

Current models for the mechanism of STAT activation of gene transcription propose that following dimerization, STAT complexes translocate to the nucleus.

However, mechanism of transport to the nucleus remains unclear, since STAT proteins lack the nuclear localization signal (NLS; Johnson et al., 1998b), which are required for nuclear transport mediated through the importin mechanism (Gorlich and Mattaj, 1996). Johnson and coauthors (1998b) proposed an intriguing model for nuclear translocation of STATs after activation by interferon gamma (IFNγ). Since the carboxy-terminal domain of the IFNγ molecule contain a NLS, they propose that following binding to IFNγ a complex containing the IFNγ-receptor, jak kinases, STATs and the bound ligand become internalized by endocytosis. Upon cytoplasmic localization, the NLS sequence in the IFNγ molecule could associate with the importin protein complex, which would then catalyze the transport of this complex to the nucleus, where STAT-mediated transcription activation would ensue. They provided evidence for actual nuclear translocation of a peptide containing the carboxy-domain of the IFNγ molecule. Although seemingly unique, they provide evidence of over 30 cytokines and/or their receptors, which utilize STATs as signal transducers that contain NLS in their sequence, indicating that this ligand-receptor-assisted nuclear translocation is a viable, and intriguing mechanism. Among such cytokines and receptors are the human IFNα and the human IFNαR1 molecules. Data in a recent paper is in variance with this concept (Milloco et al., 1999). Those authors engineered a STAT-1-estrogen receptor chimera, in which the estrogen receptor ligand binding domain was fused to the carboxy-terminus of STAT-1 molecules. After

transfection to STAT-deficient U3a cells, this "conditionally active STAT" underwent dimerization following estrogen/tamoxifen treatment. Moreover, these chimeras were able to undergo nuclear translocation and activated transcription of interferon-induced genes such as IRF-1. The authors concluded that tyrosine phosphorylation of STAT is probably only a trigger for dimerization, since dimerized, non-phosphorylated STAT chimeras also were able to stimulate interferon-specific gene activation. Furthermore, since the estrogen receptor domain used in the chimera did not contain any NLS, dimerization alone was sufficient to promote nuclear translocation, sequence-specific DNA binding and transcription activation functions of the chimeric STATs. A study conducted by Strehlow and Schindler (1998) indicated that the amino-terminal 100 amino acids of particular STATs mediated their nuclear translocation activity. Chimeric constructs in which those amino acids in STAT-1 were substituted by those of STAT-2 abolished nuclear translocation of STAT-1, while other functions were maintained, such as activation by receptor, dimerization and DNA binding. Collectively, it is fair to say that the mechanism of STAT nuclear translocation remains unclear. Although the work of Johnson et al. (1998b) puts forth an exciting proposition for such a mechanism, data from Milocco and others (1999) argues against the requirement of a ligand-receptor-assisted transport mechanism. However, existence of both mechanisms is feasible in vivo.

Interferon-directed gene activation

After translocation to the nuclear compartment, STAT complexes can act as transcription factors, to direct expression of interferon-induced genes. The best studied transcription activation complex containing STAT dimers is called interferon-stimulated gene factor 3 (ISGF-3), which is composed of a STAT 1:2 dimer and a nuclear DNA binding protein, p48 (Darnell et al., 1994; Bluyssen et al., 1996). ISGF-3 was first identified in electrophoretic mobility shift assays as a complex induced by interferon treatment. It was formed independent of protein synthesis, and was found to bind to consensus sequences on the regulatory region of interferon-stimulated genes (Kessler et al., 1988). Consensus sequences are known as interferon-stimulus response elements (ISREs). Williams and Haque (1997) present a summary of sequences of ISREs of known interferon-induced genes. A second interferon-induced transcription-activation complex also was identified and named ISGF-2 (Kessler et al., 1988). Such a complex is formed contingent on protein synthesis, presents different pattern of migration in mobility shift assays and was later identified as the transcription factor interferon regulatory factor 1 (IRF-1; Parrington et al., 1993). Interestingly, IRF-1 and p48 are from the same family of proteins and can bind to the same promoter elements (i.e., ISREs) in the regulatory region of interferon-stimulated genes (Kessler et al., 1988; Parrington et al., 1993). The p48 and STAT 1:2 dimer do not associate in a stable manner to form the ISGF-3 complex in the absence of DNA. However, contacts of amino acids 150 to 250 in the STAT-1

molecule with the carboxy-terminal portion of DNA-bound p48 stabilizes ISGF-3 (Horvath et al., 1996). Vickenmeier and coworkers (1996) reported direct binding of recombinant, tyrosine phosphorylated STAT-1:1 dimers to tandem DNA sequences. STAT-2 also forms homodimers, but requires p48 for strong transactivation of transcription (Bluyssen and Levy, 1997). However, interactions with DNA were not stable. Addition of STAT-1 increased the affinity and altered sequence selectivity of p48-DNA interactions. In this scenario, ISGF-3 assembly involves p48 functioning as an adaptor protein to recruit STAT-1 and STAT-2 to an ISRE, STAT-2 contributes with potent transactivation but is unable to directly contact DNA, while STAT-1 stabilizes the complex by contacting DNA directly. Alternatively to transcription-induction through ISRE binding, interferons also induce genes like IRF-1 which lack ISREs. Such genes are induced through sequences named Inverted Repeats, present in their promoters (Haque and Williams, 1994).

JAK-STAT pathway regulation

As in other tyrosine-phosphorylation-induced signaling systems, biological responses resulting from activation of the JAK-STAT pathway are transient (Shuai et al., 1992). Although the pathway of activation via the JAK-STAT pathway is well established, few molecules have been identified that switch the signal off (Starr and Hilton, 1999). Intuitively, one would predict that regulation of a tyrosine phosphorylation pathway could occur through the actions of phosphatases, to inactivate phosphotyrosil groups on receptors, JAKS and STATs, and proteases,

to degrade activated complexes. There is evidence for occurrence of both mechanisms of regulation in the JAK-STAT pathway (i.e., phosphatases and proteases), but more recent data indicate presence of novel regulatory molecules also playing a role. Callus and Mathey-Prevot (1998) showed that treatment of Ba/F3 cells with a specific proteasome inhibitor led to stable tyrosine phosphorylation of the interleukin-3 (IL-3) receptor and STAT-5, after stimulation with IL-3. Further investigation revealed that stable phosphorylation events were due to prolonged activation of JAKs. Moreover, Kim and Maniatis (1996) demonstrated that after activation with interferon-γ, STATs became ubiquitinated and quickly degraded. In contrast with data from Kim and Maniatis (1996), but in agreement with data from Callus and Mathey-Prevot (1998), Haspel and others (1996) reported that proteasome inhibitors increased time of activation of STAT-1 by prolonging signals from the receptor (i.e., preventing degradation of receptor-JAKs complexes), but not by blocking removal of phosphorylated STATs. This was based on the finding that ^{35}S-labeled STAT-1 translocated to the nucleus upon tyrosine phosphorylation and later returned to the cytoplasm in non-phosphorylated configuration. Data from Strehlow and Schindler (1998) agrees and expands these findings, in that chimeric STATs with mutated amino-terminal domains exhibited defects in nuclear translocation and deactivation, indicating that these two events might be linked (i.e., deactivation may be dependent on previous nuclear localization). Indeed, David and others (1993) demonstrated that a nuclear tyrosine-phosphatase is responsible for deactivation of

phosphorylated STATs. To support the existence of a mechanism for regulating activity of STATs based on phosphatases, Haque and coauthors (1995) reported that treatment of cells with orthovanadate, molybdenate and tungstate, which are effective inhibitors of protein-tyrosine phosphatases, resulted in accumulation of interferon-γ-induced phosphorylated STATs. Involvement of novel molecules in the regulation of the JAK-STAT pathway was reviewed by Starr and Hilton (1999). They propose a model in which suppressers of cytokine signaling (SOCS) proteins such as SOCS1 bind directly to JAKs to inhibit their catalytic activities. Another protein, CIS, binds to activated receptors to prevent docking of STATs. SH2-domain phosphatase-1 (SHP-1) dephosphorylates JAKs or activated receptors. Finally, a protein inhibitor of activated STAT (PIAS) inactivates STAT dimers. Song and Shuai (1998) demonstrated that SOCS 1 and SOCS3 inhibited interferon-mediated antiviral and antiproliferative activities in HeLa cells. This was linked with abolished tyrosine phosphorylation and nuclear translocation of STAT-1 in response to interferon-α. Chung and others (1997a) reported that PIAS3 directly interacted with STAT-3 and inhibited DNA binding of both STAT-3:3, STAT-1:3 dimers. Binding of STAT-1 homodimers was not affected. Moreover, cotransfections of both STAT-3 and PIAS3 showed a decrease in luciferase activity from an IRF-1 reporter gene with increasing amounts of PIAS3.

Specificity of interferon signaling

Taken together, information presented in previous sections offers several opportunities for occurrence of specific cellular responses to interferons. Such opportunities include: (1) milieu of subtypes of interferons present at the receptor, in which for example, different iso-forms of ovine IFN-τ have different abilities to extend estrous cycle length in ewes (Ealy et al., 1998); (2) composition of the receptor complex, where recruitment of particular subunits may affect which STATs are recruited; (3) amino acid context of the phosphotyrosine module on the receptor chain, and amino acid context of the SH2 domain on STATs will also determine which STAT will dock to which receptor chain; (4) which STATs are present and which dimers will form upon ligand binding; (5) mechanism of nuclear translocation of STATs, since whether STATs translocate as dimers alone or in combination with ligand-receptor complexes may influence the configuration and specificity of the transcriptional activation complex; (6) formation of single or multiple transcription activation complexes, which will depend on nature of dimers and interacting nuclear proteins; (7) dynamics of downregulation of JAK-STAT pathway, in which specific branches of the pathway may be inhibited while others may remain active to elicit specific responses; finally (8) interactions with other cellular pathways, which will be discussed next.

Cross talk with other intracellular pathways

The best known cross-talk between JAK-STAT and other signaling pathways is that represented by serine and threonine phosphorylation of STAT

residues, both constitutively and in response to ligands (see Leaman et al., 1996 for review). Such phosphorylation events are important, since treatment of cells with kinase inhibitors disrupts STAT-3:3 DNA complexes. A mitogen-activated protein kinase (MAPK) may be involved in phosphorylation of serine residues of STAT-1, because the serine 727 lies in a consensus sequence for MAPK phosphorylation. In fact, Stancato and coworkers (1997) proposed a model in which activation of MAPK was dependent on activated JAK kinases. Binding of interferon-α/β induced tyrosine phosphorylation of JAK-1, which stimulated activity of membrane bound Raf-1. Activated Raf-1 phosphorylates MEK and activates MAPK. MAPK in turn phosphorylates serine residues on STAT-1, contributing to modulation of activity for this signal transducer. However, modulation of STAT activities by MAPK may be stimulatory or inhibitory. For example, Chung and others (1997b) reported serine phosphorylation of STAT-3 by growth factors, while STAT-1 was poor substrate for several MAPK tested. Interestingly, serine phosphorylation of STAT-3 negatively modulated tyrosine phosphorylation of this protein, and consequently inhibited dimerization, nuclear translocation and gene activation.

Signal transducers such as IRS-1 and IRS-2 that are activated in response to insulin, IL-2, IL-4 etc, are tyrosine phosphorylated by JAK-1. Epidermal growth factor(EGF) is able to activate tyrosine phosphorylation of STATs 1 and 3 (David et al., 1996). Interestingly, this does not require presence of JAKs. Moreover, truncated receptor constructs containing the intrinsic kinase activity but lacking

the autophosphorylation domains were also effective in phosphorylating STATs. This indicates that an alternative mechanism, where docking through SH2 domain of STATs is not required for phosphorylation, is in place for EGF-induced STAT phosphorylation.

The obligatory intracellular bacterium of macrophages, Ehrlichia chaffeensis, blocked tyrosine phosphorylation of STAT-1, JAK-1 and JAK-2 in response to IFN-γ within 30 minutes of infection (Lee and Rikihisa, 1998). Also, PKA activity was increased 25 fold after infection. Inhibitors of PKA activity partially abrogated the E chaffeensis-induced inhibition of STAT-1 tyrosine phosphorylation, suggesting negative regulation of the JAK-STAT pathway by the PKA-dependent mechanisms.

Another interesting theme is the occurrence of synergistic effects as a result of coactivation of cellular pathways involving the JAK-STAT system. For example, cooperation of interferon-γ and tumor necrosis factor (TNF) during inflammatory responses is a result of cooperation between STAT-1 and the transcription factor NF-κβ. Synergistic expression of several genes involved in the inflammatory process was contingent on presence of both transcription factors (Ohmori et al., 1997). Stimulation by oncostatin M (OSM) induces expression of matrix metalloproteinases (MMPs). Analysis of the regulatory region of MMP-1 gene revealed presence of an AP-1 site as well as a STAT binding element. Korzus and coworkers (1997) reported enhancement of MMP

expression due to synergistic actions of AP-1 and STAT-1. Such an effect was Ras-dependent, which implies crosstalk between the MAPK and the JAK-STAT pathways of signal transduction.

Yet another example of crosstalk is between the JAK-STAT pathway and the PI 3' kinase, which has both lipid and serine kinase activities. Pfeffer and others (1997) reported that PI 3' kinase is tyrosine phosphorylated through the JAK-STAT pathway. Tyrosine phosphorylated STAT-3 proteins, bound to the IFNαR1 chain of the interferon receptor, serve as a docking site for PI 3' kinase, which couples its SH2 domain to tyrosine phosphorylated residues in the STAT molecule. Upon docking, the PI 3' kinase is activated by JAKs, which then promotes serine phosphorylation of STAT-3 to increase STAT-3 activity. In another study (Uddin et al., 1997) interferon-α stimulated serine kinase activity of PI 3' kinase, which in turn activated the signal transducer IRS-1. Moreover, stimulation with interferon β caused activation of MAPK, and such stimulation was inhibited by Wortmannin, an inhibitor of PI 3' kinase activity. This suggests involvement of the PI 3' kinase on MAPK activation. In contrast with data from Pfeffer and others (1997), wortmannin failed to inhibit formation of the ISGF3 complex and interferon-mediated induction of ISG-15, indicating that the PI 3' kinase probably is not required for interferon effects.

Finally, the work of Flati and others (1996) indicates that stimulation of cells with interferon-α causes activation of PLA_2, as measured by release of AA in

culture medium. PLA_2 was associated with JAK-1, and inhibitors of PLA_2 activity prevented formation of active ISGF3 transcription complexes. However, such inhibition did not block binding of activated STAT-1 to inverted repeat sequences, such as present in the regulatory region of IRF-1. Moreover, treatment of cells with interferon-α stimulates tyrosine phosphorylation of PLA_2. The authors argue for a structural role of PLA2, which may be required for correct assembly of the ISGF3 transcription complex.

The JAK-STAT pathway in bovine endometrium

In addition to the bIFN-τ receptor data mentioned above, very little has been done on elucidation of the signal transduction system activated by bIFN-τ in the endometrium. In fact, one of the main objectives of this dissertation was to provide evidence of existence, as well as details on the bIFN-τ -activated, endometrial JAK-STAT pathway.

Spencer and others (1998) conducted two studies to detect induction of interferon-stimulated transcription factors, IRF-1 and IRF-2. Both factors were absent in cyclic ewes and present in pregnant ewes (days 11 and 13, cyclic and days 13, 15 and 17, pregnant). In cyclic ewes with ligated uterine horns, unilateral infusion ovine IFN-τ induced expression of IRF-1 and IRF-2 but not the uterine horn receiving a BSA infusion. Since expression of these factors is contingent on a functional JAK-STAT pathway, these data support existence of such a pathway in the endometrium. Bathgate and coworkers (1998) also

reported existence of IRFs in endometrium of pregnant cows. Perry and coauthors (1999) reported presence of STATs 1 and 2 and IRF-1 in the nucleus of BEND cells stimulated with bIFN-τ. More detailed evidence for the JAK-STAT pathway (i.e., existence, tyrosine phosphorylation, nuclear translocation, dimer formation of and gene activation via STAT proteins) in bovine endometrium is presented in Chapters 4, 5 and 6.

Bovine bIFN-τ -simulated protein synthesis in the endometrium

A main proposition of this dissertation is that proteins synthesized or suppressed as a result of activation of the JAK-STAT pathway interact with the $PGF_{2\alpha}$ synthesizing machinery to inhibit $PGF_{2\alpha}$ secretion in the endometrium. In this section, I will describe the data available on proteins synthesized in the endometrium in response to bIFN-τ and their possible influences in the $PGF_{2\alpha}$ system.

Rueda and coworkers (1993) reported secretion of 12 and 28 kD proteins both from pregnant endometrial explants and cyclic endometrial explants stimulated with bIFN-τ in vitro. In a subsequent paper, Naivar and others (1995) further characterized those proteins and discovered a novel, 16 kDa secretory protein (P16). Endometrium explants were obtained from day 18 pregnant cows and incubated in presence or absence of bIFN-τ. Both basal and stimulated secretion of all three proteins increased in culture medium in a time-dependent manner. More importantly, the 12 kD protein (Rueda et al., 1993), now renamed

as P8, was induced only in response to bIFN-τ, but not in response to IFN-α, suggesting the possibility of bIFN-τ eliciting specific signal transduction and protein synthesis. Moreover, P8 but not P16 secretion could be stimulated by phorbol ester (Staggs et al., 1998). Amino acid analysis of the P8 revealed identity with the alpha chemokine family: 92-100% identity with bovine bGCP-2 (Teixeira et al., 1997). Functions of bGCP-2 remain elusive, but it has been suggested (Hansen et al., 1999) that being a chemokine, bGCP-2 may attract conceptus cells to attachment sites in the endometrium. Also, bGCP-2 may attract cells from the immune system, to release cytokines beneficial to embryonic development. P16 was identified as a bovine ubiquitin-cross reactive protein (Austin et al., 1996a,b). The bUCRP mRNA (Hansen et al., 1997) and protein (Austin et al., 1996b) are induced by bIFN-τ, and sequence analysis of the bUCRP gene revealed presence of a conserved ISRE in the promoter region, indicating putative activation by bIFN-τ (Perry et al., 1997). Analysis of the primary structure of bUCRP revealed presence of critical amino acids and domains implicated in functions of ubiquitin, such as conjugating with other proteins. However, bUCRP lacked residues required for targeting proteins to proteasomal degradation (Austin et al., 1996a). Therefore, it was proposed that a possible role for bUCRP was to modify uterine proteins during early pregnancy (Hansen et al., 1999). In fact, Johnson and others (1998a) reported that specific conjugates of bUCRP and endometrial cytosolic proteins were formed in

response to treatment with bIFN-τ. Moreover, such complexes were distinct from complexes containing ubiquitin, indicating a bIFN-τ-induced, specific action. Although proteins present in the bUCRP conjugates have not yet been identified, an attractive hypothesis is that bIFN-τ induces conjugation of bUCRP to proteins involved in the cascade of $PGF_{2\alpha}$ production in the endometrium. Such targeting could modify function of such proteins to make them less able to stimulate $PGF_{2\alpha}$ production.

Research from Spencer and coworkers (1998) also showed that endometrial estrogen receptors and oxytocin receptors were reduced in the uterine horns infused with ovine IFN-τ, and this was negatively correlated with observed increase in IRF-1 and IRF-2 expression. Since IRF-2 has been implicated as an inhibitor of gene transcription (Harada et al., 1994), the authors hypothesized that perhaps interferon-induced IRFs were involved in inhibition of gene transcription for estrogen and oxytocin receptors. In fact, Fleming and coworkers (1998) cloned the ovine estrogen receptor gene and discovered IRF response element (IRE) consensus sequences in the promoter region, further supporting the hypothesis of interferon modulation of estrogen receptor expression. Deletion constructs of the estrogen receptor promoter linked to luciferase reporter gene were transfected into endometrial cells. Treatment of these cells with ovine IFN-τ caused reduction in luciferase expression only in constructs containing the IREs. Using the same rationale, Bathgate and others

(1998) sequenced the bovine oxytocin receptor gene and also found IREs in the regulatory region, and such sites bound bovine IRF-1 and -2. Again, the suggestion is that perhaps bIFN-τ-induced transcription repressors may downregulate expression of oxytocin receptors, to ultimately decrease $PGF_{2\alpha}$ secretion in the pregnant uterus.

Hypothetical model for bIFN-τ -mediated suppression of $PGF_{2\alpha}$ secretion in the endometrium

The hypothetical model shown in Figure 2-3 depicts some of the possibilities discussed thus far.

Uterine-Conceptus Interactions and Reproductive Failure in Cattle

Thus far, this review has illustrated the enormous amount and intricacy of interactions that need to occur between embryonic and maternal uterine tissues in order for a successful pregnancy to be established. Given the high percentage of embryonic mortality occurring during early pregnancies, it becomes apparent that a precise program of interactions must be followed, and that deviations from such a program may lead to pregnancy termination. Such a program includes both embryonic and maternal components. For example, the embryonic unit must be able to effectively interact with maternal endometrium, undergo elongation and send antiluteolytic signals to the maternal unit in order to survive. The maternal unit should provide a quiescent and nutritive environment,.

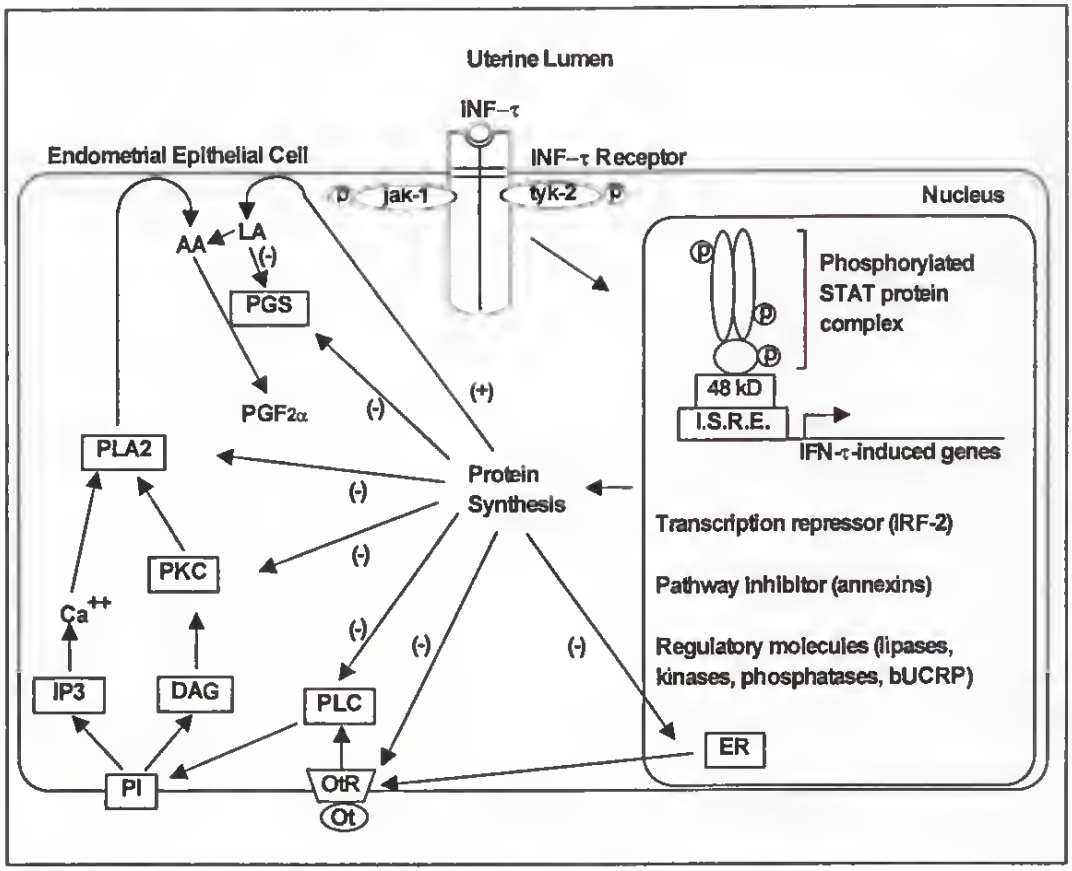


Figure 2-3. Hypothetical model of interferon-τ (IFN-τ)-stimulated gene activation and effects on molecules involved in the $PGF_{2\alpha}$ synthetic pathway. Estrogen receptors (ER) are up-regulated before luteolysis and stimulate synthesis of oxytocin (OT) receptors (OTR). Oxytocin binding to OTR stimulates phospholipase C (PLC), which cleaves phsphatydilinositol (PI) yielding inositol trisphosphate (IP_3) and diacylglycerol (DAG). The IP_3 stimulates release of Ca^{++} from intracellular stores, and DAG activates protein kinase C (PKC). The PKC activates phospholipase A2 (PLA_2) which, in the presence of Ca^{++}, cleaves arachidonic acid (AA) from membrane phospholipids. Molecules of AA and linoleic acid (LA) regulate the enzyme prostaglandin synthase (PGS) to produce $PGF_{2\alpha}$. In the pregnant cow, embryonic trophoblastic cells secrete IFN-τ into the uterine lumen. Receptors on endometrial epithelial cells bind IFN-τ, and dimerize. Dimerization of receptors promote phosphorylation (represented by a circled "p") of associated tyrosine kinases from the JAK family, such as tyk-2 and jak-1. Phosphorylated receptors attract signal transducer and activators of transcription (STAT) proteins. The STAT proteins are phosphorylated in tyrosine residues by the JAK kinases and form a complex that migrates to the nucleus. In the nucleus, the complex associates with a 48 kD DNA-binding protein, and this new complex binds to interferon-stimulated response elements (ISRE) in the regulatory region of interferon-induced genes, activating transcription of such genes and synthesis of proteins. Synthesized proteins may act to specifically block one or more steps on the $PGF_{2\alpha}$ synthetic pathway (arrows with [-] signs; see text for details and abbreviations).

conducive for embryonic attachment, and should bear intracellular mechanisms to receive and transduce antiluteolytic signals from the conceptus that ultimately inhibit the default, $PGF_{2\alpha}$-secretory pathway of the uterus. Thatcher and Hansen (1992) reported that day 17 conceptuses varied in size from 15 to 250 mm. Since inhibition of $PGF_{2\alpha}$ is probably dependent on total amount of bIFN-τ secreted and on area of endometrium occupied by the conceptus, smaller conceptuses would have already a smaller chance of survival. Environmental effects such as heat stress (discussed above) decrease conceptus development and apparently compromises ability of the conceptus to secrete bIFN-τ, leading to failure in pregnancy recognition. There is also evidence for a role of the uterus to stimulate secretion of bIFN-τ by conceptus. Hernandez-Ledezma and coworkers (1992) cultured IVF (in vitro fertilization)-produced embryos to blastocyst stage and either continued in vitro culture or transferred conceptuses to synchronized recipient cows. Embryos were recovered 4 days later, placed in culture dishes and secretion of bIFN-τ was quantified. Secretion of bIFN-τ was highly stimulated by exposure to the uterine environment, indicating that optimal production of the antiluteolytic signal by the conceptus is not solely determined by the conceptus. Stojkovic and coworkers (1999) reported that bovine embryos derived by embryo flushing and in vitro production produced more bIFN-τ in long term culture than embryos derived from nuclear transfer or embryo splitting Such differences may contribute to lower pregnancy rates following embryo transfer to

recipients. Failure of cows to extend CL lifespan in response to bIFN-τ have been reported (Helmer et al., 1989b; Meyer et al., 1995). This indicates failure in the interferon receptor system, JAK-STAT-mediated signal transduction, post-signaling mechanisms within the endometrium or a combination of these factors. These responses have not been examined in a population of cows and warrant further investigation.

Manipulating Uterine Function to Minimize Embryo Mortality

Bovine IFN-τ administration

Based on the variation of conceptus size and consequent capacity to secrete at the critical time of maternal recognition of pregnancy for CL maintenance, it is reasonable to propose that supplementing bIFN-τ at that critical period may decrease embryonic losses. The rationale is that a slightly underdeveloped conceptus that may be unable to deliver the appropriate antiluteolytic signal may be rescued by exogenous bIFN-τ administered at around day 17 after insemination. Lack of availability of recombinant bIFN-τ and structural similarity with bIFN-α prompted Newton and others (1990) to test fertility effects of bIFN-α. Interferon-α extended CL lifespan but caused side effects such as increased body temperature. Barros and coworkers (1992) conducted a field experiment where bIFN-α was administered daily from days 14 to 17 of pregnancy or as a single injection on day 13. Conception rates were

actually decreased by about 10% compared to control animals. This was attributed to bIFN-α-induced side effects such as hyperthermia and acute drops in P_4 concentration. Alternative delivery systems and use of actual bIFN-τ may still make this technology useful in the field (see Thatcher et al., 1994a for discussion).

Fat feeding

Another possible manipulation of this system consists of attempting to make the uterus less luteolytic, by changing the proportion of luteolytic AA to antiluteolytic linoleic acid. For example, Thatcher and others (1997) described an experiment in which Menhaden fish meal was fed to cows for 25 days. Fish meal contains both eicosapentanoic and docosahexaenoic fatty acids, which had been shown to be able to decrease $PGF_{2\alpha}$ secretion. Indeed, cows fed fish oil had a much attenuated secretion of PGFM in response to an oxytocin challenge.

Prevention of heat stress

One single environmental challenge that has negative effects both in the embryonic and maternal units during the period of maternal recognition of pregnancy is heat stress, as discussed previously. Therefore, strategies to reduce effects of high environmental temperatures, such as observed in tropical and subtropical latitudes, warrant investigation and application.

Objectives of This Dissertation

1) To study the distribution pattern of oviductal secretory proteins secreted by cows bearing persistent or fresh dominant follicle;

2) To examine the signal transduction system stimulated by bIFN-τ in endometrium;

3) To characterize the effects of bIFN-τ on $PGF_{2\alpha}$ production by BEND cells.

CHAPTER 3
PERSISTENT DOMINANT FOLLICLE ALTERS PATTERN OF OVIDUCTAL SECRETORY PROTEINS FROM COWS AT ESTRUS

Introduction

Synchronization of the estrous cycle in cattle is a very important tool for reproductive management. For example, synchronization systems are used widely for artificial insemination, timed insemination and embryo transfer. Most commonly, synchronization is achieved with combinations of treatments with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), progestins and GnRH (Thatcher et al., 1996). Synchronization with progestins is based on the principle that exogenous progestins, such as progesterone delivered by a Controlled Internal Drug Release (CIDR) device, can maintain a sub-luteal concentration of progestin in blood during a period which permits CL regression. In the absence of a CL, removal of the progestin source will result in a synchronized estrus (Macmillan and Peterson, 1993).

During the estrous cycle in cattle, two to three follicular waves of dominant follicle development occur (Savio et al., 1988; Sirois and Fortune, 1988). Each follicular wave is comprised of periods of recruitment, selection, dominance and turnover or atresia. The ovulatory follicle generated in the last wave does not

turn over, but ovulates in a low progesterone (P_4) environment. Turnover of the dominant follicle (DF) is associated with high concentrations of P_4, typical of mid-cycle, which lowers LH pulse frequency (Kinder et al., 1996). Turnover of the first wave DF can be blocked by exogenous progestins and injection of $PGF_{2\alpha}$ (Cooperative Regional Research Project, NE-161, 1996; Savio et al., 1993a; Savio et al., 1993b). The resulting sub-luteal concentration of progestin in plasma permits an increase in LH pulse frequency which sustains growth of the DF. This "persistent" DF (PDF) is estrogenic, and subsequent fertility, as measured by conception rate at first service (number of pregnancies / number of animals inseminated), is lower compared to animals bearing normal DFs [37.1% vs. 64.8% in heifers, (Savio et al., 1993b); 23.6% vs. 58.2% for cows and heifers, (Cooperative Regional Research Project, NE-161, 1996). Fertility after AI, however, is restored to levels comparable to controls if the PDF is turned over and a freshly recruited follicle is allowed to ovulate. Possible explanations for reduced fertility include alterations in the oocyte and /or in the oviductal environment. In a study by Ahmad et al. (1995), cows ovulating a PDF had embryos that at day 6 of pregnancy were less developed (i.e., were less able to reach the 16-cell stage) than embryos from cows ovulating a fresh (F) DF. In addition, Revah and Butler (1996) showed that oocytes recovered from the PDF showed expanded cumulus cells and condensed chromatin dispersed in their ooplasm. In contrast, compact cumulus cells and intact germinal vesicles were found in oocytes from FDF. Thus, the PDF may affect oocyte maturation,

oviduct and uterine function, which could affect early embryonic development and decrease fertility.

Macromolecules present in oviductal fluid have been suggested to serve an important role in sperm capacitation (Anderson and Killian, 1994), fertilization (Boatman and Magnoni, 1995) and early embryo development (Gandolfi et al., 1989). Therefore, alterations in oviductal biosynthetic activity including protein synthesis and secretion may affect conception rate.

Steroid modulation of oviductal synthesis and secretion of proteins has been characterized in sheep (Buhi et al., 1991, Murray, 1993), baboon (Verhage and Fazleabas, 1988) and swine (Buhi et al., 1989; Buhi et al., 1990). An altered steroid environment, associated with development of a PDF, may alter oviductal protein synthesis and secretion. In turn, the altered pattern of protein synthesis and secretion could affect optimal oviductal function, fertilization and early embryo development that contributed to reduced embryonic survival in synchronized cows. The present experiment tested the hypothesis that the presence of a PDF alters protein synthesis and secretion of oviductal explants from cows at estrus.

Specific objectives were: 1) to induce a PDF or a FDF with the strategic use of $PGF_{2\alpha}$, progesterone-containing CIDR and GnRH; 2) to compare the biosynthetic activity and the array of secretory proteins synthesized in the infundibulum (INF), ampulla (AMP) and isthmus (IST) at estrus in oviducts

ipsilateral (IPSI) and contralateral (CONTRA) to the DF of cows bearing a PDF versus a FDF.

Materials and Methods

Materials

Impervo paint was from Benjamin Moore and Co. (Jacksonville, FL) and All-weather Paintstick was from LA-CO Industries, Inc./Markal Company (Chicago, IL). Donations of Lutalyse were made by Pharmacia-Upjohn Co. (Kalamazoo, MI), Buserelin from Hoescht-Roussel Agri-Vet (Somerville, NJ) and CIDR-B devices were donated by EAZI-BREED™, InterAg (Hamilton, New Zealand). Eagles' minimum essential medium (MEM, catalog number 86-5007), non-essential aminoacids (100x), anti-mycotic/antibiotic solution (100x) and MEM vitamin solution (100x) were from Life Technologies (Gibco Laboratories, Grand Island, NY). L-[4,5-^{3}H] leucine (leu; 159 Ci/nmol) was from Amersham Life Sciences, Inc. (Arlington Heights, IL) and L-leu, L-methionine, L-glutamine, D(+) glucose, bovine pancreatic insulin, riboflavin and molecular weight standards were purchased from Sigma Chemical Co. (St. Louis, MO). Spectra/por 3 dialysis membrane was from Spectrum Medical Industries Inc. (Houston, TX). Acrylamide, NN'-methylenebisacrylamide, sodium dodecyl sulphate, Nonidet-P40, urea, agarose, diallyltartardiamide were from BDH Laboratory Supplies (Poole, England). Ampholines were from Pharmacia

(Uppsala, Sweden), TEMED and ammonium persulphate were from Bio-Rad (Hercules, CA). Glycine was from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). Coomassie brilliant blue, fast green, bromophenol blue, β-mercaptoethanol, hydrochloric acid, sodium hydroxide, tris (hydroxymethyl) aminomethane, sodium salicylate, acetic acid and chromatography paper were from Fisher Scientific (Fairlawn, NJ) and X-OMAT x-ray film was from Eastman Kodak Co. (Rochester, NY).

Preparation of Medium

Leucine-deficient modified minimum essential medium (MEM; 10% normal concentration of leu) was prepared as described by Buhi and coworkers (Buhi et al., 1990). Briefly, MEM was supplemented with glucose (3g/l), methionine (1.5 mg/l), leu (5.2 mg/l), sodium bicarbonate (2.2 g/l), MEM vitamins (10 ml/l), non-essential amino acids (10 ml/l), insulin (7.41 mg/l), sterile filtered and adjusted to pH 7.4. Before use, medium was supplemented further with glutamine (292 mg/l), methionine (13.5 mg/l) and antimycotic-antibiotic solution (10 ml/l).

Animals and Treatments

During the pre-treatment period, estrous cycles of six mature non-lactating cows were synchronized (Figure 3-1). A used CIDR device containing approximately 1.2 g (Van Cleef et al., 1992) of P_4 was placed into the vagina of

each cow for 7 days. One day prior to CIDR removal, cows received an injection

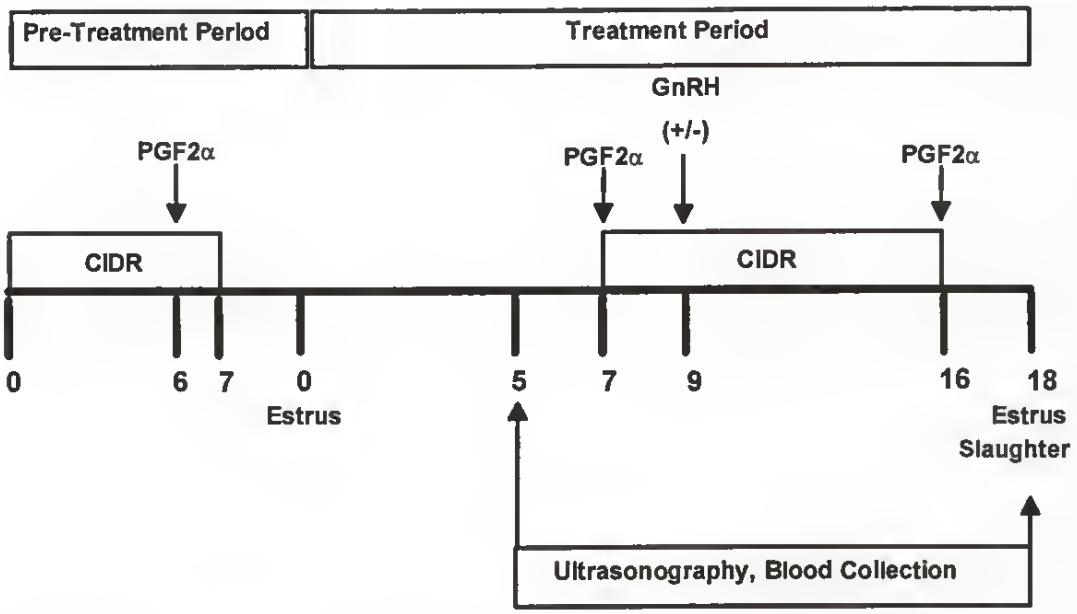


Figure 3-1. Experimental protocol (see text).

of prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$, Lutalyse, 25 mg) to regress the CL. To aid with estrus detection, tail heads were painted (Impervo) and chalked (All-weather Paintstick). Cows were observed twice daily for signs of estrus, and paint scores were assigned (Macmillan et al., 1988). The day of standing estrus was designated experimental day 0. During the treatment period, ovaries were examined by transrectal ultrasonography using an Aloka echo camera model SSD 500 linear array ultrasound scanner equipped with a 7.5MHz transducer (Aloka Co., Japan). From Days 5 to 18, follicles and CL were measured daily and sizes recorded. In addition, blood samples were collected in heparinized evacuated tubes (Vacutainers, Becton Dickson Vacutainer System USA,

Rutherford, NJ) by tail venipuncture and stored in an ice bath. Plasma was harvested by centrifugation (1800 x g for 30 minutes) and stored at -20°C until assayed for estradiol-17β (E_2) and P_4. On day 7, all cows were injected with $PGF_{2\alpha}$ and received one used CIDR device (Savio et al., 1993b). On day 9 cows were assigned randomly to one of two treatment groups. Cows of the FDF group (n=3) received an injection of GnRH agonist (Buserelin, 8 mg), to induce turnover of any large size follicles present at that time, and allow recruitment of fresh follicles (Schmitt et al., 1996c). Cows of the PDF group (n=3) did not receive the GnRH agonist. On day 16 CIDR devices were removed, and cows received an injection of $PGF_{2\alpha}$ (25 mg). Cows were checked for signs of estrus twice daily and slaughtered when observed in standing estrus (day 18 or 19). The experimental models for persistent and fresh follicles resulted in a greater pregnancy rate for heifers inseminated at estrus induced by FDF (Savio et al., 1993b; Schmitt et al., 1996c).

Tissue Culture

On the day of slaughter, reproductive tracts were removed aseptically, and oviducts were identified as IPSI or CONTRA to DF, dissected, trimmed free of mesosalpynx and divided into INF, AMP and IST regions based on gross anatomical characteristics. Segments of tissue between IST and AMP were discarded. Tissue from each region was cut longitudinally to expose the lumen, and then minced into fragments of ≈50mm^3. Tissue fragments from each

functional region were cultured (Buhi et al., 1990) in LEU-deficient minimal essential medium supplemented with ^{3}H-LEU in the ratio of 100 mg tissue/3 mL medium/20 mCi ^{3}H-LEU for 24 hours at 37°C in a controlled atmosphere of N_2:O_2:CO_2 (50%:47.5%:2.5% by volume). For AMP and INF, 500 mg of tissue were cultured per dish, while for IST variable amounts of tissue (between 140 and 290 mg) were used.

Two-Dimensional Electrophoresis

After 24 hours incubation, conditioned media were dialyzed extensively (MW cut-off 3500) against Tris buffered saline (10 mM Tris, 150 mM NaCl) pH 7.6 (two changes of 4 liters each/24 hours) and then dialyzed against deionized water (two changes of four liters each/24 hours). Radioactivity in the retentate was determined by liquid scintillation spectrometry, and incorporation rate was defined as dpm non-dialyzable macromolecules/mg wet tissue. For each sample, a volume of dialyzed conditioned medium containing 4 x 10^5 DPM was lyophilized and submitted for two-dimensional SDS-PAGE as previously described(Buhi et al., 1991). Gels were stained with Coomassie blue, soaked in 1 M Na salicylate solution, dried and exposed to x-ray film for 35 days at -80°C.

Densitometry

Fluorographs were developed, and after qualitative analysis 20 protein spots were selected and analyzed quantitatively by densitometry (AlphaImager

2000, Alpha Innotech Corporation, San Leandro, CA). Since a constant amount of DPM was loaded for all samples, the capacity of tissues to synthesize and secrete macromolecules (DPM/mg of tissue) was not accounted for and, therefore, unadjusted densitometric measurements were biased. Different secretory capacities were corrected by expressing the densitometric measurements per unit secretory tissue. In this way, densitometric measurements from tissues with greater secretory capacity were adjusted upwards and vice versa for tissues with lower secretory capacities. Adjustments were calculated by the equation: adjusted Arbitrary Density Units (ADU) = ADU/mass of tissue equivalents, where one tissue equivalent is the mass of tissue needed to synthesize and secrete 4×10^5 DPM of labeled macromolecules. Mass of tissue equivalents was obtained by dividing 4×10^5 DPM by incorporation rate (DPM non-dialyzable macromolecules/mg of tissue) for individual tissue samples.

Hormone Assays

Concentrations of E_2 and P_4 in plasma were measured by radioimmunoassays previously validated in our laboratory [E_2:(Badinga et al., 1992); P_4:(Knickerbocker, 1986)]. Intra- and inter-assay coefficients of variation were 15.5 and 12.4%, respectively, for E_2 and, 6.8 and 8.1%, respectively, for P_4

Statistical Analysis

Data were analyzed by least squares analysis of variance using the General Linear Models of Statistical Analysis System (SAS, 1988). Concentrations of E_2 and P_4 in plasma and diameter of DF were analyzed by split plot ANOVA. The mathematical model used treatment (FDF or PDF), cow (treatment), day, treatment by day and error. Rate of incorporation of radioactivity into oviductal tissue and natural Log of adjusted ADU measurement of proteins were calculated and analyzed by least squares ANOVA. The mathematical model was: treatment (FDF or PDF), cow (treatment), side (IPSI or CONTRA to the DF), region (INF, AMP and IST), all higher order interactions and error. Orthogonal contrasts for treatment (PDF vs. FDF), region (INF and AMP vs. IST and INF vs. AMP), and treatment by region interactions were used to compare means.

Results

Ultrasonography and Hormone Measurements

Size of DF was analyzed in two phases during the treatment period: from day 5 to day 9 (period prior to injection of GnRH) and from day 10 until day 16 (Figure 3-2). Both FDF and PDF cows had similar sizes of DF from day 5 to day 9. However, a significant ($p<0.01$) treatment by experimental day interaction was

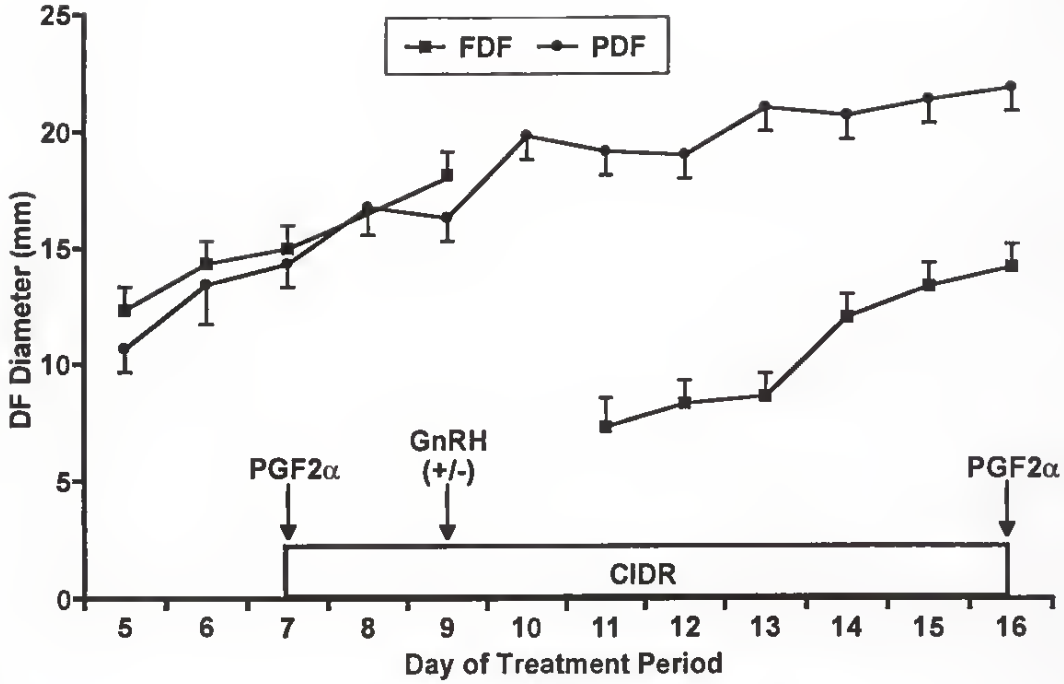


Figure 3-2. Least squares means (± SEM) of **diameter of the dominant follicle** (DF) of cows bearing a fresh DF (FDF, treated with GnRH on d 9) or persistent DF (PDF, not treated with GnRH on d 9) during the Treatment Period. Treatments with $PGF_{2\alpha}$, CIDR and GnRH are indicated. Day 0 represents day of estrus at the beginning of Treatment Period.

detected from day 10 to day 16. All cows with FDF ovulated the first wave DF and a newly recruited DF was detected on day 11 which reached 12 mm by day 16. In contrast, the first wave DF of PDF group was sustained and reached a size of 22 mm by day 16.

Concentrations of E_2 (Figure 3-3, panel a) and P_4 (Figure 3-3, panel b) were analyzed between experimental day 7 (day of $PGF_{2\alpha}$ injections) and day 18 or 19. There was a significant ($p<0.01$) treatment by experimental day interaction for both E_2 and P_4 concentrations in plasma. After GnRH injection on day 9, E_2 concentrations decreased in plasma of FDF cows and remained between 5 and 10 pg/ml until day 16 and increased to 22 pg/ml at day 18

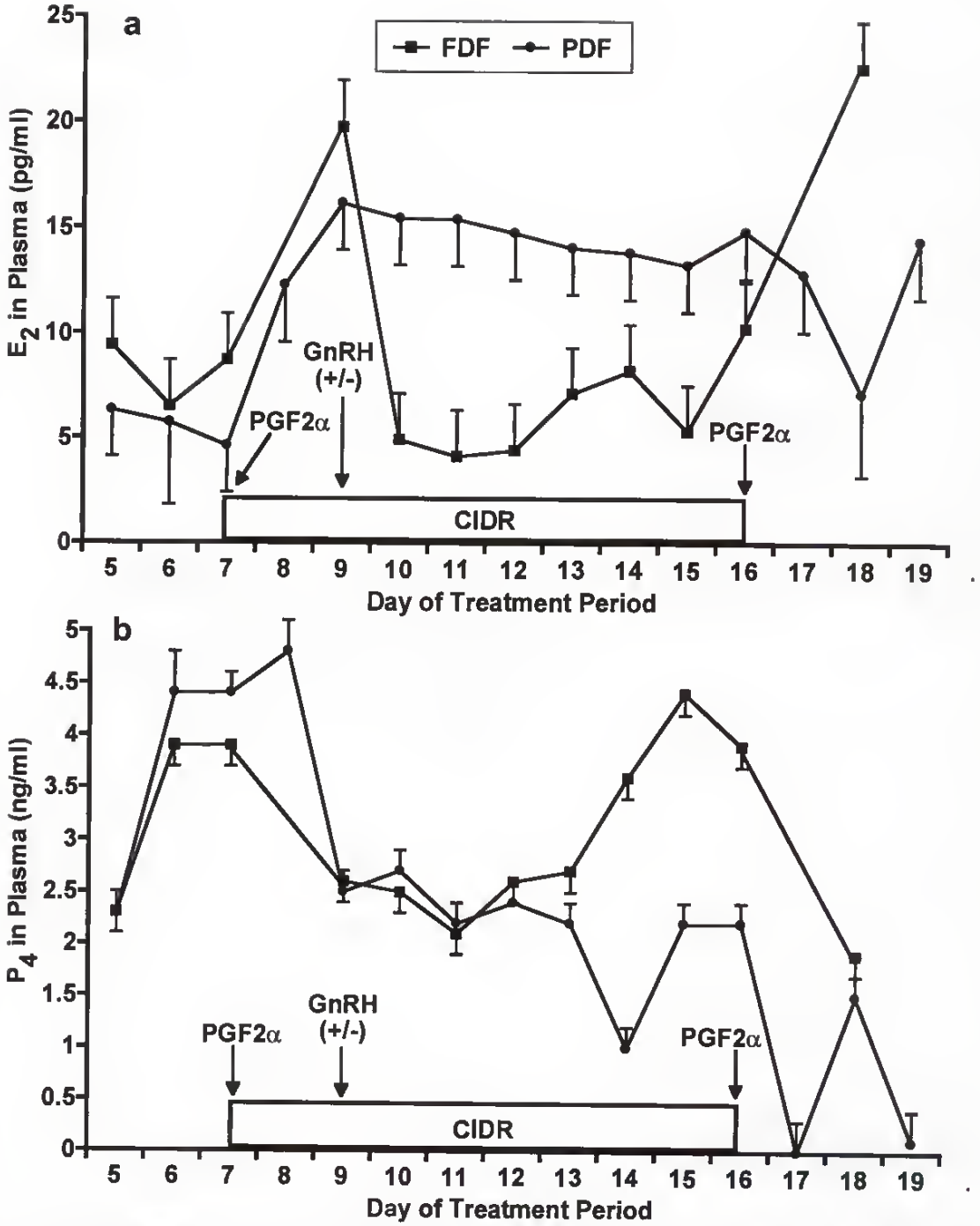


Figure 3-3. Least squares means (± SEM) of **concentrations of ovarian steroids in plasma** of cows bearing a fresh dominant follicle (FDF, treated with GnRH on d 9) or persistent dominant follicle (PDF, not treated with GnRH on d 9) during the Treatment Period. Treatments with $PGF_{2\alpha}$, CIDR and GnRH are indicated. Day 0 represents day of estrus at the beginning of Treatment Period.

a) **estradiol-17β** (E_2); b) **progesterone** (P_4).

(estrus). For PDF cows, E_2 remained at approximately 15 pg/ml from day 9 until day 19. After $PGF_{2\alpha}$ injection on day 7, P_4 concentrations decreased for both groups between day 7 and day 11. After day 11, P_4 increased in association with development of a new CL in FDF cows (3/3) while concentrations of P_4 remained low for PDF cows. After CIDR removal and $PGF_{2\alpha}$ injection on day 16, P_4 concentrations decreased for FDF and PDF cows.

Incorporation Rate

Incorporation rate of radiolabel into protein can be used as a measure of the protein biosynthetic activity of tissues (i.e., amount of ^{3}H-leu incorporated into newly synthesized and secreted macromolecules). There was a significant ($p<0.05$) treatment by region interaction (Figure 3-4). The FDF increased incorporation rate of ^{3}H-leu into proteins for all oviductal regions (treatment effect; $p<0.01$). However, stimulation was not significant in the IST (INF and AMP vs. IST by treatment contrast $p<0.01$). No side or side by treatment effects were detected ($p>0.1$).

Fluorography and Densitometry

The pattern of proteins secreted by explants of INF, AMP and IST, as resolved by two-dimensional SDS-PAGE, are shown in representative fluorographs in Figure 3-5. Proteins analyzed were designated P1 to P20 based on their location in the fluorograph following a clockwise pattern starting in the

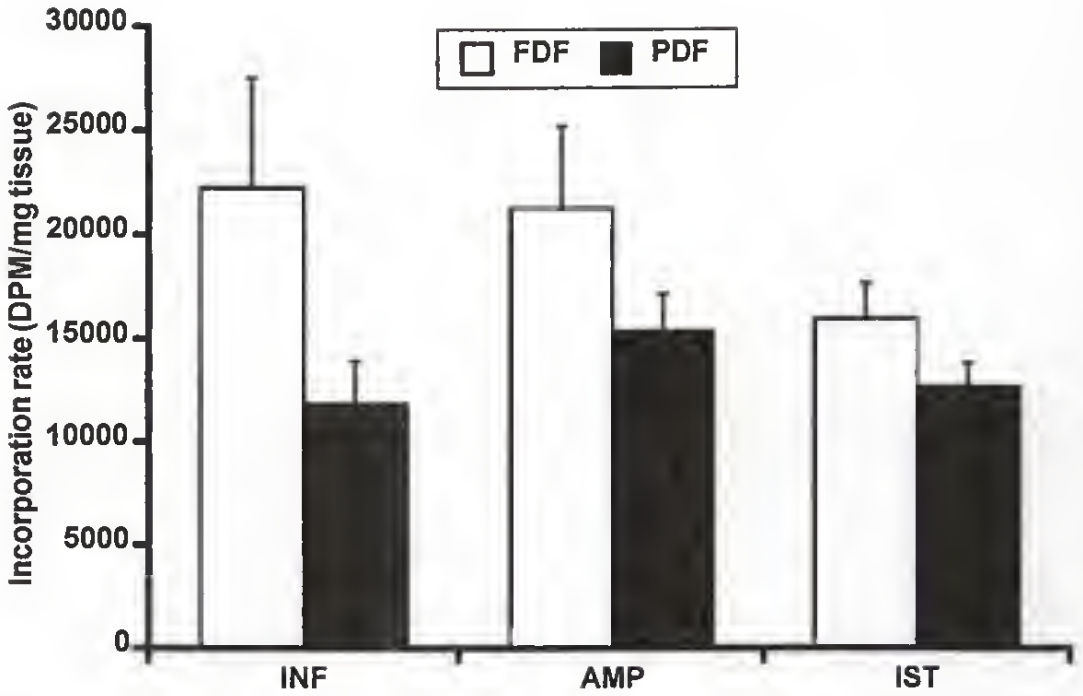


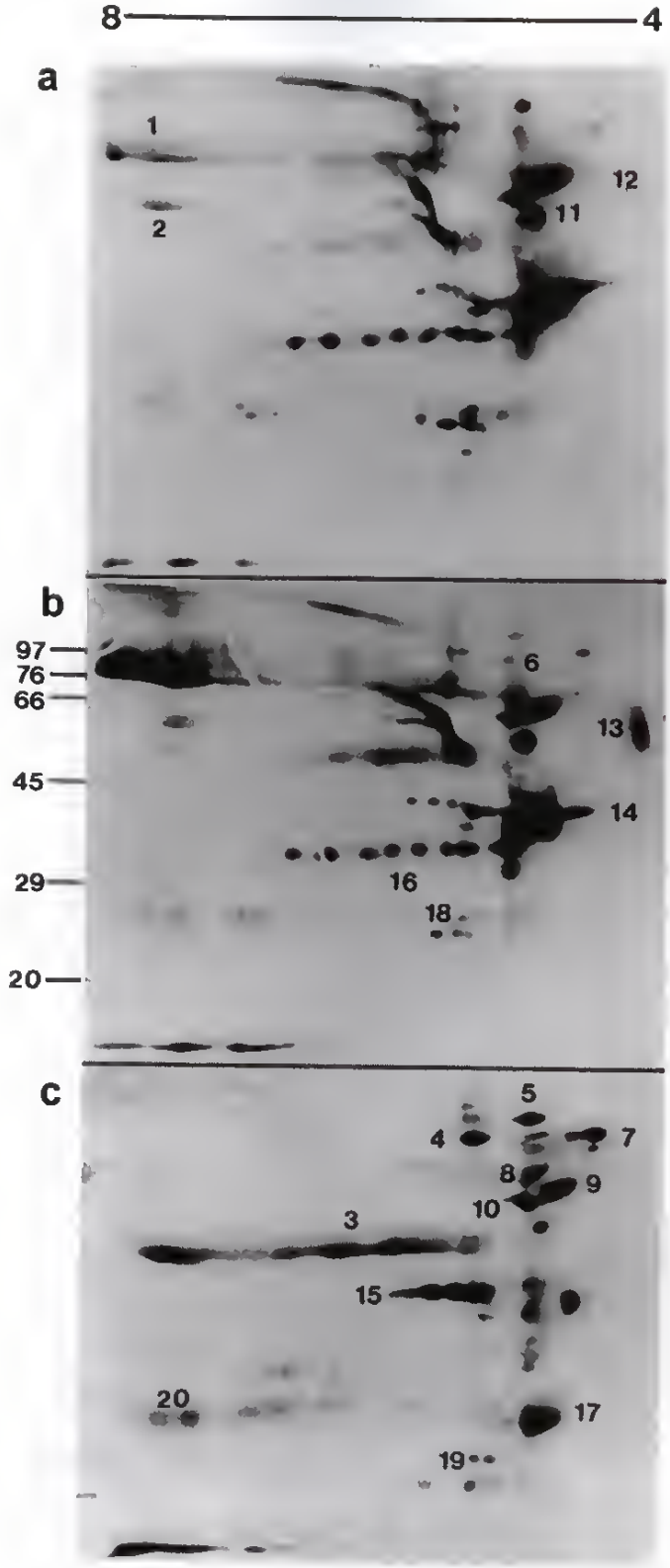
Figure 3-4. Least squares means (± SEM) of **Incorporation rates of ^{3}H-leucine** into infundibulum (INF), ampulla (AMP) and isthmus (IST) of cows bearing a fresh dominant follicle (FDF, treated with GnRH on d 9) or persistent dominant follicle (PDF, not treated with GnRH on d 9) during the Treatment Period.

top left quadrant. Results of densitometric analyses of P1 to P20 are depicted in Tables 1 and 2.

There was a trend for effect of treatment for P2 (p<0.06) and P13 (p<0.07). Interpretation of main effects of treatments on secretion of proteins indicate that presence of FDF stimulated greater secretion of P2 and P13 compared to tissues from PDF cows. There was a significant (p<0.05) region effect for P1, P4-7, P12-17 and P20, and a tendency (p<0.1) for P2, P3, P11 and P18. Effects of side were significant for P5 (p<0.05) and P19 (p<0.05), and approached significance for P8 (p<0.1) and P15 (p<0.06). Region by treatment

Figure 3-5. Representative fluorographs of two-dimensional SDS-PAGE analysis of culture medium conditioned by oviductal explants. Proteins analyzed by densitometry (1 through 20) are indicated. Molecular weight standards are indicated ($X10^{-3}$), and pH gradient runs from left (pH 8) to right (pH 4).

a) Infundibulum; b) Ampulla; c) Isthmus.


8
4
a
1
2
12
11
b
97
76
66
45
29
20
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18
c
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20
17
19

TABLE 1: Relative molecular weights (MW[a]) and least squares means (LSM) and probability (*p*) values of the logarithm of densitometric units of region by treatment interactions of selected proteins (P) on 2-D gels[b].

SPOT	MW	INF-FDF	INF-PDF	AMP-FDF	AMP-PDF	IST-FDF	IST-PDF	reg.by trt.
P1	82±3	9.7±.3	9.3	10.4	10.1	7.4	8.7	$p<0.07$
P2	67±5	7.9±1	8.3	7.9	6.7	5.7	4.5	NS[c]
P3	58±2	6.4±1.1	7.9	6.6	6.9	8.9	9.2	NS
P4	97±5	6.4±.2	5.8	5.9	5.9	6.8	6.6	NS
P5	76±4	6.2±.1	6.2	5.9	6.4	6.6	6.9	NS
P6	95±5	6.5±.2	6.4	5.8	6.0	6.5	6.8	NS
P7	97±5	5.3±.7	5.4	1.1	5.8	6.7	6.9	$p<0.02$
P8	80±4	7.3±.1	7.1	7.4	7.2	7.0	7.1	NS
P9	76±4	7.6±.1	7.7	7.9	7.7	7.6	7.8	NS
P10	72±3	7.5±.1	7.4	7.6	7.5	7.5	7.6	NS
P11	65±4	7.1±.1	7.4	7.8	7.6	7.3	7.4	NS
P12	71±3	3.5±1	4.9	2.1	.5	4.1	4.8	NS
P13	68±4	6.2±2	3.6	4.7	0	0	0	NS
P14	47±3	6.2±1.2	6.2	6.2	0	0	0	$p<0.02$
P15	47±2	5.2±1	6.1	5.6	7.1	9.0	8.8	NS
P16	37±2	7.4±1	7.5	9.1	8.7	2.2	5.6	NS
P17	27±2	7.0±.3	6.3	6.4	6.0	8.1	7.7	NS
P18	27±2	6.9±.2	6.8	6.3	6.2	6.4	6.4	NS
P19	21±1	5.9±1.7	5.2	4.5	5.4	6.4	6.3	NS
P20	28±1	9.0±.2	8.4	8.2	8.2	8.7	8.9	NS

a: Relative molecular weight (Mr x 10^{-3}; means ± S.E.M.).
b: LSM ± pooled S.E.M. of region (reg.; INF = infundibulum; AMP = ampulla; IST = isthmus) by treatment [trt.; FDF = fresh dominant follicle (DF); PDF = persistent DF] interactions.
c: Non significant ($p>0.1$).

TABLE 2: Relative molecular weights (MW[a]) and least square mean (LSM) and probability (*p*) values of the logarithm of densitometric units side by treatment interactions of selected proteins (P) on 2-D gels[b].

SPOT	MW	IPSI-FDF	CONTRA-FDF	IPSI-PDF	CONTRA-PDF	side by trt.
P1	82±3	9.2±.2	9.4	9.4	9.3	NS[c]
P2	67±5	7.2±.6	7.5	7.0	5.9	NS
P3	58±2	5.4±.9	7.4	8.2	7.7	NS
P4	97±5	6.1±.17	6.5	6.2	6.0	NS
P5	76±4	5.9±.2	6.5	6.5	6.5	*p*<0.06
P6	95±5	5.8±.1	6.5	6.5	6.3	*p*<0.05
P7	97±5	3.1±.5	4.5	6.3	5.8	NS
P8	80±4	7.0±.1	7.5	7.2	7.2	*p*<0.07
P9	76±4	7.6±.1	7.9	7.8	7.7	*p*<0.05
P10	72±3	7.4±.11	7.7	7.5	7.5	NS
P11	65±4	7.2±.1	7.6	7.5	7.4	*p*<0.03
P12	71±3	3.0±.7	2.9	4.8	2.0	NS
P13	68±4	3.7±.8	2.7	.3	0	NS
P14	47±3	2.8±1.2	3.7	1.4	0.7	NS
P15	47±2	7.8±.9	4.0	8.4	6.4	NS
P16	37±2	6.9±1	5.8	6.2	8.3	NS
P17	27±2	6.9±.2	7.3	6.7	6.6	NS
P18	27±2	6.4±.2	6.6	6.4	6.5	NS
P19	21±1	5.9±.9	2.9	5.9	5.4	*p*<0.09
P20	28±1	8.4±.2	8.7	8.5	8.5	NS

a: Relative molecular weight (Mr x 10^{-3}; means ± S.E.M.).
b: LSM±pooled S.E.M. of side (CONTRA = contralateral to DF; IPSI = ipsilateral to DF) by treatment interactions.
c: Non significant ($p>0.1$).

interactions were significant for P7 and P14 ($p<0.02$), and tended to be significant for P1 ($p<0.07$). As suggested by significant treatment by region interactions, protein synthesis and secretion in response to treatments varied according to region. In the AMP, P7 was stimulated by PDF whereas P14 was stimulated in FDF cows. Protein 14 was present in the INF and absent in the IST regardless of treatment, but ampullary P14 was abolished by PDF. In the IST, synthesis of P1 was stimulated by PDF. Proteins with significant side by treatment interactions were P6 ($p<0.05$), P9 ($p<0.05$), P11 ($p<0.03$), while P5 ($p<0.06$), P8 ($p<0.07$), and P19 ($p<0.09$) only approached significance. Treatment by side interactions indicate a differential response of IPSI and CONTRA sides to FDF compared to PDF. For FDF cows, abundance of P5, P6,P8, P9 and P11 was reduced in the IPSI compared with the CONTRA side. In contrast, abundance of these same proteins was similar across sides for PDF cows. Protein 19 was secreted in similar amounts at the IPSI side for both treatments. In the CONTRA side however, PDF maintained, while FDF reduced abundance of P19 compared to the IPSI side. Treatment by side by region interaction was significant for P19 ($p<0.01$) and tended to be significant for P2 ($p<0.1$) and P13 ($p<0.06$).

A summary of mean comparisons of effects of treatment, side, treatment by region and treatment by side for individual spots is presented in Table 3.

TABLE 3: Summary of means comparison of effects of treatment[a], treatment by region[b] side[c] and treatment by side interactions on the logarithm of densitometric units of selected proteins (P) in two-dimensional gels[d].

Effect	Comparison	Proteins
Treatment	FDF>PDF	P2, P13
Treatment by region	AMP-FDF>AMP-PDF	P14
	AMP-PDF>AMP-FDF	P7
	IST-PDF>IST-FDF	P1
Side	CONTRA>IPSI	P7, P8
	IPSI>CONTRA	P15, P19
Treatment by side	CONTRA-FDF>CONTRA-PDF	P6, P8, P9, P11
	CONTRA-PDF>CONTRA-FDF	P19
	IPSI-PDF>IPSI-FDF	P5, P6, P9, P11

a: FDF=fresh dominant follicle (DF); PDF=persistent DF.
b: INF=infundibulum; AMP=ampulla; IST=isthmus.
c: IPSI=ipsilateral to CL; CONTRA=contralateral to CL.
d: Proteins are listed for effects which were different with $p<0.1$.

Discussion

In the present study, we have shown that hormonal manipulations of animals altered both their follicular and luteal functions, which provided a model to study oviductal protein synthesis and secretion at estrus from distinctly different periestrus hormonal environments (Figs. 2 and 3). Distinctly different steroidal environments for cows bearing PDFs or FDFs specifically modulated

biosynthetic activity and protein synthesis and secretion from different functional regions of the oviduct. This altered pattern of protein synthesis and secretion in cows bearing PDFs may contribute to the lower fertility of this group of animals compared to cows ovulating FDFs (Savio et al., 1993b). Normal embryos transferred 7 days after estrus to uteri of cows that ovulated a PDF had no difference in pregnancy rates compared to controls (Wehrman et al. 1997). This supports the concept that low fertility associated with PDF may be due to an inappropriate oviductal, not uterine, environment before day 7, or an abnormal embryo (Ahmad et al., 1995; Mihm et al., 1994; Revah and Butler, 1996). Both ultrasonography and hormonal data indicated that the first wave DF of the experimental period was ovulated after injection of GnRH agonist at day 9 (FDF cows) while no injection of GnRH agonist (PDF cows) permitted sustained development of the first wave DF. Continued growth of the first wave DF occurred in the low P_4 environment supported by the CIDR in the PDF group. The PDF maintained high concentrations of estradiol in plasma. Cows of the FDF group ovulated the first wave DF in response to GnRH, and the resulting CL secreted increasing amounts of P_4 after day 13. A newly selected FDF was detected by day 11. Changes in E_2 and P_4 between PDF and FDF groups reflected the differences in CL and follicle dynamics have been reported previously (Schmitt et al., 1996c). The acute increase in E_2 concentration in the FDF group was associated with development of a DF during the pro-estrous period following injection of $PGF_{2\alpha}$ and withdrawal of the CIDR.

These endocrine environments induced distinctly different patterns of protein synthesis and secretion by all oviductal regions, between cows with PDF vs. FDF. Studies in sheep (Buhi et al., 1991), baboon (Verhage and Fazleabas, 1988) and pigs (Buhi et al., 1989; Buhi et al, 1990; Buhi et al., 1997b) indicate that specific oviductal proteins and specific mRNAs are regulated by endogenous steroids during the estrous cycle or early pregnancy and by exogenous steroids in ovariectomized animals. In the pig, a family of related glycoproteins (POSP 1-3), a basic and an acidic 100,000 Mr protein and a very acidic protein (75,000-85,000 Mr), are synthesized primarily by the AMP during proestrus, estrus and metestrus (high E_2, low P_4) but not diestrus (low E_2, high P_4). Consistent with POSP protein synthesis and secretion, POSP mRNA expression is also estrogen-dependent and significantly greater in the AMP on day 0 and day 1 of estrous cycle or pregnancy (Buhi et al., 1996). Similar to POSP, the cow oviduct produces a basic 97 kD protein (Boyce et al., 1990) identified as P1 in this study. Protein 1 secretion reaches a maximum at estrus and decreases during the luteal phase of the estrous cycle. In agreement with Boice et al. (1990), P1 was secreted by all oviductal regions in the present study. Other proteins, such as tissue inhibitor of metalloproteinase-1, found to be produced by the pig and cow oviduct (P20 in this study) was shown to be expressed optimally on day 2 in the pig, when E_2 and P_4 were both low (Buhi et al., 1992). Further, E_2 appeared to suppress synthesis and/or secretion of several protein complexes which were not identified (Buhi et al., 1992).

Therefore, it was expected that the sustained high E_2/low P_4 milieu of PDF cows and the increasing E_2 and P_4 milieu of FDF cows would differentially modulate protein synthesis and secretion in the oviduct.

Analysis of the biosynthetic activity (incorporation rates) indicated that presence of a PDF decreased the synthetic activity of oviductal tissues. However, the overall increased incorporation of label into synthesized and secreted macromolecules for FDF cows compared to PDF cows in all oviductal regions appears to be in variance with the similar abundance of specific proteins for both PDF and FDF cows (Tables 1 and 2). Several explanations for this dichotomy are possible. Higher E_2 and lower P_4 concentrations associated with presence of a PDF over an extended period of time, apparently had a suppressive effect on overall biosynthetic activity of the different oviductal regions. Prolonged exposure to high levels of E_2 in PDF cows may have caused down-regulation of E_2 receptor, which would explain the suppression in biosynthetic activity of oviducts from PDF cows compared to FDF cows. However, synthesis and secretion of proteins that are actually inhibited by E_2 could be stimulated as a result of down-regulation of E_2 receptors. Studies in the hen (Kawashima et al., 1996), mouse (Fuentealba, 1988), pig (Stanchev et al., 1985) and primates (Slayden and Brenner., 1994) indicate that absolute and relative amounts of receptors for E_2 and P_4 vary in the oviduct, during the estrous cycle and pregnancy. Moreover, oviductal functions such as velocity of egg transport (Fuentealba et al., 1988) and oviductal epithelial cell proliferation

(Slayden and Brenner, 1994) also change in response to manipulations of the steroid environment and steroid binding to their receptors. Therefore, concentrations of circulating E_2 and P_4 for FDF and PDF cows may have differentially regulated numbers of E_2 and P_4 receptors in the oviduct, and consequently, expression of steroid-responsive proteins (as illustrated by changes in oviductal function). Alternative explanations for the discrepancy between incorporation rate and abundance of specific protein spots are: (1) in FDF cows, incorporation may have been greater in proteins of higher (>97kD) or lower (<20 kD) Mr which were not resolved and would be undetectable in gel analyzes; or, (2) FDF may have induced a higher turnover of proteins and the resulting partially degraded proteins were not resolved by electrophoresis (i.e., MW between 3.5 and 20 kD).

Several studies in cattle suggest the importance of the oviductal region and protein milieu in reproductive processes. In studies with bulls (Anderson and Killian, 1994), it has been demonstrated that culture medium conditioned by IST tissue at estrus capacitated more sperm than did medium conditioned by AMP. This increase was abolished by heating the conditioned medium and inactivating proteins before incubation with sperm. Staros and Killian (1998) showed that four unidentified oviductal proteins and a P1-like protein from non-luteal oviductal fluid would associate with the zona pellucida, suggesting a modulation of sperm/egg binding or embryonic development by oviduct-derived proteins. In the present experiment, of 20 proteins analyzed by densitometry, P2

and P13 showed differences in synthesis between treatments. Both proteins were inhibited by PDF, suggesting an overall down-regulation. The strong effect of region for 16 of 20 spots measured suggested a biosynthetic gradient in which the secretion was greater or less for the IST depending upon the protein. Such a gradient has been reported in the pig and sheep (Buhi et al., 1992; Buhi et al., 1996; DeSouza and Murray, 1995; Murray, 1993). Moreover, DeSouza and Murray (1995) reported differential secretion of a chitinase-like protein, similar to P1 in response to steroid treatments in sheep, while Buhi et al. (1996) showed differential expression POSP mRNA among oviductal regions in pigs.

Treatment by region interactions indicate that the steroid milieu generated by PDF vs. FDF modulated synthesis and secretion of particular proteins differently, depending on the oviductal region. As an example, PDF abolished synthesis of P14 in the AMP, while it was absent in the IST and present in the INF regardless of the treatment. In contrast, P7 synthesis was induced by PDF in the AMP, although P7 was present in similar amounts for both treatments in the INF and IST. It is likely that optimal function of each region is achieved when the oviductal micro-environment includes the appropriate secretory proteins.

Treatment by side interactions indicated that the oviduct adjacent to the ovary bearing the DF responded differently depending upon follicular status (FDF vs. PDF). Ireland et al. (1984) demonstrated that blood drainage from the ovary containing the DF contained higher concentrations of E_2 compared to the CONTRA ovary. Exposure to higher concentrations of E_2 may therefore

preferentially alter synthesis of selected proteins. In the present study, P5, P6, P8, P9 and P11 were reduced in the IPSI side compared to the CONTRA side in FDF cows, whereas for PDF cows, abundance was similar regardless of the side. This indicates that PDF overrode the side-dependent regulation of secretion of P5, P6, P8, P9 and P11 that occurs normally in FDF cows. Perhaps sustained exposure to E_2 downregulates E_2 receptors that abolished the decrease in protein secretion associated with E_2.

In summary, FDF and PDF regulation of protein synthesis and secretion in the oviduct is protein, region and side specific. This suggests that several mechanisms are involved in the complex regulation of oviduct function. Possible mechanisms include: E_2 and P_4 receptor regulation; differential action of E_2 and P_4 (i.e., stimulatory vs. inhibitory) depending on protein, side and region; effects of autocrine and paracrine factors; cross-talk between E_2- and P_4-induced signal transduction and other intracellular effector systems.

In addition to steroids, it is possible that other effectors may control oviductal protein synthesis and secretion. A low P_4 environment elicited by a progestin-containing device in PDF cows is associated with higher LH pulse frequency compared to FDF cows (5, 6). Derecka et al. (1995) reported the presence of LH receptor mRNA in porcine oviduct tissue. Recently, LH receptor protein and mRNA transcripts were described in bovine oviductal epithelial cells (1997). Moreover, these authors reported that hCG treatment of bovine oviductal epithelial cells in vitro induced time- and dose-dependent secretion of a

95 kD oviductal glycoprotein. Therefore, it is possible that different patterns of LH release may directly affect the oviduct and modulate differential protein synthesis in FDF compared to PDF cows.

The present study identified a series of proteins in which synthesis and secretion are modulated differentially according to exposure of the oviduct to the in vivo steroid milieu. This indicates that the oviductal micro-environment is altered. Collectively, our findings add support to the concept that a less than optimal oviductal micro-environment may contribute to the low fertility of cows bearing a PDF. We propose that a combination of the effects of premature maturation of oocytes and inappropriate oviductal micro-environment is responsible for the decreased fertility observed in cows bearing PDF.

This chapter provides clear evidence for alterations on maternal-embryonic communications occurring in the oviduct that could lead to disruption of the reproductive cycle. Remaining chapters will examine maternal-conceptus communications during maternal recognition of pregnancy for maintenance of the CL.

CHAPTER 4
EFFECTS OF BOVINE INTERFERON-TAU ON THE JAK-STAT SIGNAL TRANSDUCTION PATHWAY AND SYNTHESIS OF PROTEINS IN BOVINE ENDOMETRIUM AND ON THE MECHANISM OF GENERATION OF PROSTAGLANDIN F2α IN ENDOMETRIAL EPITHELIAL CELLS

Introduction

Bovine (b) conceptuses secrete copious amounts of the glycoprotein interferon-tau (IFN–τ) around day 17 of pregnancy (Farin et al., 1990). Bovine IFN–τ suppresses luteolytic pulses of prostaglandin F2α ($PGF_{2\alpha}$) to rescue the maternal corpus luteum (CL) from luteolysis (Thatcher et al., 1997). Therefore, bIFN–τ is considered to be a mediator to maintain pregnancy. This Chapter describes experiments designed to examine two separate aspects of bIFN-τ effects in the bovine endometrium, namely (1) signal transduction and protein synthesis and (2) effects on the $PGF_{2\alpha}$-synthesizing machinery.

Trophoblasts secrete bIFN–τ into the uterine lumen and bIFN–τ binds to receptors on the surface of endometrial cells (Hansen et al., 1989; Li and Roberts, 1994). Binding of bIFN–τ elicits intracellular events that stimulate synthesis and secretion of specific proteins in bovine endometrial explants (Naivar et al, 1995) and decreases secretion of $PGF_{2\alpha}$ by endometrial cells

(Danet-Desnoyers et al., 1994). However, the nature of such intracellular events within the endometrium is unknown. Interferon-alpha (IFN-α) shares sequence homology with bIFN–τ (Roberts et al., 1992), efficiently competes with bIFN–τ for binding to endometrial epithelium (Li and Roberts 1994), and also has antiluteolytic effects in cattle (Plante et al., 1989; Plante et al., 1991). Studies with human cells showed that IFN-α stimulates transcription of IFN-responsive genes through phosphorylation of specific transcription factors identified as "Signal Transducers and Activators of Transcription" (STAT) proteins (STAT-1, 84/91 kD; STAT-2, 113 kD; Darnell et al., 1994; Fu et al., 1992; Schindler et al., 1992). Moreover, ovine IFN–τ also stimulates transcription in Daudi cells through phosphorylation of STAT proteins (Subramaniam et al., 1995). It is hypothesized that bIFN–τ stimulates bovine endometrial cells through a similar signal transduction mechanism. An initial series of experiments was designed (1) to verify that bIFN–τ stimulated synthesis of specific intracellular and secretory endometrial proteins; (2) to examine whether STAT proteins are present in bovine endometrium; (3) to test whether bIFN–τ causes phosphorylation of STAT proteins in endometrial explants.

Arnold and coworkers (1999) incubated endometrial explants from cows infused in vivo with bIFN-τ or a control protein (bovine serum albumin, BSA) with intracellular stimulators of $PGF_{2\alpha}$ synthesis. They found that in vivo treatment with bIFN-τ inhibited the ability of phorbol 12, 13 dibutyrate (PDBu) and a

calcium ionophore to stimulate secretion of $PGF_{2\alpha}$, compared to in vivo infusion with BSA. Intracellularly, PDBu and the calcium ionophore stimulate activity of protein kinase C (PKC, Silvia and Homanics, 1988; Silvia et al., 1994). The PKC promotes phosphorylation and activation of phospholipase A2 (PLA_2, Parker et al., 1987; Lin et al., 1993), which cleaves arachidonic acid (AA) from phospholipids in the cellular membrane (Lin et al., 1992). Arachidonic acid is a substrate for cyclooxygenase-2 (COX-2), which directs AA to prostaglandin synthesis (Smith et al., 1992). In order to suppress secretion of $PGF_{2\alpha}$ in response to those treatments, bIFN-τ must interact with the $PGF_{2\alpha}$ synthesizing machinery in the endometrium. In a second experiment, objective was to test the hypothesis that uterine infusions of bIFN-τ decrease abundance of PLA_2 and COX-2 in endometrial epithelial cells.

Materials and Methods

Materials

Materials, equipment and reagents utilized for estrous cycle synchronization, tissue culture, electrophoresis, densitometry and fluorography were as described for Chapter 3, except when specified. Bovine IFN-τ (200 μg/ml in 20 mM Tris-HCl, pH 8; 1.08×10^7 units of antiviral activity)was a gift from Dr. Michael Roberts from the University of Missouri. Urea and Nonidet P40 were

from BDH Laboratory Supplies (Poole, England). Tissue culture-treated plastic petri dishes (100x20) were purchased from Corning (Corning Glass Works, Corning, NY). Tris, Tris-HCl, NaCl, EDTA, NaF, glycerol, glycine, methanol, gelatin, Tween 20 and 15 ml polypropylene conical tubes, Tris-HCl, EDTA, Hepes, 15 ml polypropylene conical tubes were purchased from Fisher Scientific (Pittsburgh, PA). Microcentrifuge tubes (1.5 mL) were from USA Scientific (Ocala, FL). Aprotinin, leupeptin, pepstatin, $Na_4P_2O_7$, EGTA, β–glycerophosphate, Na_3VO_4, benzamidine, PMSF and Protein-A agarose beads, bovine serum albumin, Earle's balanced salts and Hank's balanced salts (HBSS) were from Sigma Chemical Co. (St. Louis, MO). Anti-STAT-1 (E-23; catalog number SC-346, 200μg/ml) and -STAT-2 (C-20; catalog number SC-476, 200μg/ml) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-phosphotyrosine (PY-20, 1 mg/ml) antibody was from Transduction Laboratories (Lexington, KY). Nitrocellulose membranes (Hybond - ECL) and Enhanced Chemiluminescence Kit were from Amersham, Buckingamshire, England, and X-ray films were from NEN Research Products (Reflection; Boston, MA) and from Eastman Kodak Co. (X-Omat Blue XB-1; Rochester, NY). Non-fat dried milk was from Mid-America Farms (Springfield, MO), and β-mercaptoethanol was from Bio-Rad (Hercules, CA). Naxcel® was from Pharmacia & Upjohn (Kalamazoo, MI). MEM essential and non-essential amino acids solutions were from Gibco BRL (Life Technologies, Grand Island, NY).

Scalpel blades (number 20) were from Feather Safety Razor Co., Ltd, (Japan). Donations of Lutalyse were made by Pharmacia-Upjohn Co. (Kalamazoo, MI).

Experiment 1

Animals. Multiparous non-lactating dairy cows (n=3, 24 hour incubation experiment; n=3, 2 hour incubation experiment) were synchronized with one injection of $PGF_{2\alpha}$ and observed for estrus, using chalk-assisted estrus detection as described in Chapter 3 (Macmillan et al., 1988). Transrectal ultrasonography was used to confirm ovulations (48 hours after standing heat) and presence of a healthy CL prior to slaughter (day 16 of the synchronized estrous cycle) using an Aloka echo camera model SSD 500 linear array ultrasound scanner equipped with a 7.5MHz transducer (Aloka Co., Japan). At day 16 (2 hour incubations) or day 17 (24 hour incubations) of the estrous cycle, a blood sample was collected from each cow by tail venipuncture and cows were slaughtered at the Meat Laboratory of the Department of Animal Science, University of Florida. Reproductive tracts were removed immediately and transported to the laboratory on ice. Ovaries were examined for presence of CL and follicles. Endometrium was dissected from myometrium using scissors, and strips of tissue were placed in a 100x20 plastic tissue culture-treated petri dish containing modified minimum essential medium [(MEM, prepared as described in Chapter 3, but supplemented with 46.8 mg/l leucine (leu)] to prevent dehydration. Explants of endometrium

(500 mg) were further cut into five or six pieces and cultured for either 2 or 24 hours.

24 hour cultures. To study both intracellular and secretory proteins stimulated by bIFN-τ, endometrial explants were assigned randomly to two treatments (0 or 50 ng/mL bIFN-τ, diluted in medium to a volume of 100 µl which was added to wells) in duplicate and cultured for 24 hours with leu-deficient MEM supplemented with ^{3}H-leu (500 mg tissue/5 ml medium/100 mCi ^{3}H-leu) at 37°C in a controlled atmosphere of N_2:O_2:CO_2 (50%:47.5%:2.5% by volume). Dose of bIFN-τ used was slightly higher than estimates of the Kd of the ovine IFN-τ receptor for binding of ovine IFN-τ (0.2 - 11.6 ng/ml; see Danet-Desnoyers et al., 1994). After 24 hours, explants were separated from medium. Explants were homogenized to provide samples of endometrial intracellular proteins; culture medium was dialyzed to provide samples of secreted proteins.

Endometrial explants were washed in culture medium, blotted dry and stored at -70 C until homogenization. Frozen explants were placed in a 10 ml glass beaker, which was inserted half way into a plastic 100 mL beaker containing ground ice. Explants were homogenized in 2 ml of ice-cold homogenization buffer (9.3M urea, 50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 50 mM NaF, 20 mM β-glyceryl phosphate, 2 mM Na_3VO_4, benzamidine [5 mg/mL], 1 mM PMSF, 10% v/v glycerol and 1% v/v NP-40) using a Polytron tissue homogenizer (Brinkmann, Luzern, Switzerland),

intermittently pulsed for 5 seconds every 10 seconds, for a total of 1 minute, or until most tissue was homogenized and no large tissue fragments were visible. Homogenates were transferred to a 15 ml polypropylene conical tube and kept on ice. Homogenates were centrifuged in a refrigerated Sorvall centrifuge (model RC-3B, equipped with a H-6000A rotor, Du Pont Co., Wilmington, DE) at 5000 RPM for 10 minutes. Supernatants were transferred to microcentrifuge tubes and spun for 15 minutes at 14000 RPM in a microcentrifuge (model 235b, Fisher, Pittsburgh, PA) at 4C. Supernatants were dialyzed as described in Chapter 3 and concentration of radioactivity measured in aliquots from dialyzed extracts and volumes corresponding to 500000 DPM were lyophilized (Labconco Corp., Kansas City, MO, connected to a vacuum pump, Precision, model D150, Chicago, IL), reconstituted with 1X Laemmli buffer (Laemmli, 1970; 62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% v/v glycerol, 5% v/v β-mercaptoethanol, 0.001% v/v bromophenol blue) and stored at -20 C until SDS-PAGE.

Culture medium was dialyzed, as described in Chapter 3, to remove unincorporated ^{3}H-leu and degraded peptides. Concentration of radioactivity in the dialyzed samples was determined. Volumes of each dialyzed sample containing 500000 DPM were lyophilized, reconstituted in 1X Laemmli buffer and stored at -20C.

2 hour cultures. To examine presence and phosphorylation of STAT proteins 1 and 2 in the endometrium, explants were pre-incubated in medium alone for 2 hours and then randomly assigned to receive 0 or 625 ng/mL bIFN-τ

(diluted in medium to a volume of 100 μl which was added to wells), in duplicate, for 3, 10, 30, 60 or 120 minutes. Amounts and proportions of medium and tissue were the same as described for 24-hour cultures. Treatments were stopped by quickly rinsing explants in fresh medium and immediately immersing in a 8 oz. Styrofoam cup containing liquid nitrogen. Frozen tissues were stored at -70 C until homogenization. Frozen tissues were homogenized as described above, but a different buffer was used. Whole cell extract (WCE) buffer was comprised of 50 mM Tris (pH 8.0), 300 mM NaCl, 20 mM NaF, 1mM $Na_3 VO_4$, 1mM $Na_4P_2O_7$, 1mM EDTA, 1mM EGTA, 1mM DTT, 0.5 mM PMSF, 10% v/v Glycerol, 0.5% v/v NP-40 and 10μg/ml each aprotinin, leupeptin and pepstatin. Tissues were homogenized in 3 ml of ice-cold WCE buffer. Homogenates were incubated in ice for 15 minutes and clarified by centrifugation as described above to obtain WCE. Extracts were pre-cleared to reduce competition for protein A in Sepharose beads between endogenous immunoglobulins in extracts and antibodies used for immunoprecipitation (see below). Pre-clearing was performed by incubating 1 mL WCE with 40 μl protein A slurry (see below) in a microcentrifuge tube at 4 C, in a rotating device ("Roto-torque", heavy duty rotator, Cole Parmer, Chicago, IL; lever setting at "low", speed setting at 7) for 2 hours. The extract was spun in the microcentrifuge for 10 seconds and pre-cleared supernatant transferred to a clean tube. Removal of endogenous immunoglobulins was verified by incubating pellet (protein A beads) with 1 X Laemmli buffer for 5 minutes at 95 C and submitting supernatant to SDS-PAGE .

as described below. Coomassie blue staining gels displayed bands at molecular weights coinciding with heavy and light change of immunoglobulins. Protein content of pre-cleared WCE was determined using the Bradford method for protein determination (Bio-Rad, Hercules, CA). Extracts were stored at -70 C until further analysis.

SDS-PAGE and Flourography. Proteins in lyophilized culture medium and homogenized tissues from 24 hour cultures were separated by electrophoresis in 10% (w/v; intracellular proteins) or 15% (w/v; secretory proteins) polyacrylamide mini-gels, using a mini-gel apparatus (Mini-protean II, Bio-Rad, Hercules, CA; 150V, 100mA for 50 minutes). Polyacrylamide gels were prepared for fluorography as described in Chapter 3; gels were dried on a slab gel drier (model SE 1150, Hoeffer Scientific Instruments, San Francisco, CA; 1.5 hours at 60 C) and exposed to X-ray films for 15 (intracellular proteins) or 28 (secretory proteins) days at -70C.

Immunoprecipitation and immunoblotting analyses. Dried protein-A agarose beads (5 mg/sample) were swollen in 15 ml of 50 mM Tris-HCl pH 6.8 for 1 hour, washed thrice in the same buffer, transferred to a microcentrifuge tube, resuspended in 200 µl WCE buffer, and incubated with anti-STAT-1 and -STAT-2 antibodies (1 µg each/sample) for 2 hours at 4 C in a rotating device. Beads were then washed thrice in 1 ml WCE buffer and resuspended in 25 µl WCE buffer to produce a 1:1 slurry (i.e., 50% swollen beads, 50% WCE buffer). Each wash consisted of a pulse spin in a microcentrifuge to collect beads at the

bottom, removal and discard of supernatant, addition of 1 ml ice-cold WCE buffer, and resuspension of beads in this buffer by gently hand mixing. Fifty µl of slurry were added to 4000 µg of protein per sample. WCE buffer was added up to a final reaction volume of 1 ml. Samples were immunoprecipitated overnight at 4 C in a rotating device, washed thrice in WCE buffer and once with 62.5 mM Tris-HCl pH 6.8. After this last buffer was removed, tubes were pulse-spun once more and residual liquid was gently aspirated with a 25 gauge needle (PrecisionGlide®, Becton Dickinson and Co., Franklin Lakes, NJ) attached to a tuberculin syringe (Becton Dickinson and Co., Franklin Lakes, NJ). Sixty µl of 1x Laemmli buffer was added in each tube, gently mixed with the tip of a pipette and proteins were solubilized by incubating beads for 5 minutes in a heating block (Dry bath incubator, Fisher Scientific, Pittsburgh, PA) at 100 C. Samples were pulse-spun in a microcentrifuge and supernantants were used for immunoblots.

Immunoblots. Immonoprecipitated proteins were separated in 7.5% acrylamide mini-gels by 1-dimensional SDS-PAGE. Mini-gels were incubated in Towbin transfer buffer (25 mM Tris, 192 mM Glycine, 20% methanol) and proteins transferred to nitrocellulose membranes using a mini-gel transfer tank apparatus (Hoeffer Scientific Instruments, TE series transphor electrophoresis unit, San Francisco, CA) for 4 hours at 1000V and 200mA. After transfer, membranes were blocked for 2 hours in 200 ml of 2% (w/v) gelatin in Tris-buffered saline (TBS, 10 mM Tris, 150 mM NaCl, pH 7.6) supplemented with

0.1% v/v Tween-20 (TBST). Membranes were quickly rinsed twice, then washed for 15 minutes in TBST and incubated for 1 hour in 10 ml of a 1:1000 dilution of anti-phosphotyrosine monoclonal antibody (PY-20) in 2% (w/v) gelatin in TBS, quickly rinsed twice, washed 4 times for 10 minutes in TBST, incubated for 1 h in 10 ml of a 1:8000 dilution of horse radish peroxidase (HRP)-linked anti-mouse IgG antibody [part of the Enhanced Chemiluminescence(ECL) kit] in TBST containing 2% (w/v) gelatin. The membrane was quickly rinsed twice, washed once for 15 minutes and four times for 5 minutes with TBST. Membranes were allowed to drip-dry and then were exposed to ECL kit reagents (8 ml total volume), following the manufacturer's instructions. Membranes were drip-dried again and placed face down on a piece of plastic wrap, which was wrapped around them. Wrapped membranes were placed in a X-ray film cassette (Kodak X-Omatic cassette, Eastman Kodak Co., Rochester, NY) and X-ray films were exposed for 10 minutes to detect tyrosine-phosphorylated proteins. Exposed X-ray films were developed in a Konica -X-ray film processor model QX-70 (Konica Corp., Japan). Since both tyrosine phosphorylation and abundance of STATs needed to be measured in the same sample (i.e., same membrane), membranes were stripped of antibodies used to detect phosphorylated proteins and re-used for a second immunoblot, to determine abundance of STATs. Membranes were stripped in 200 ml solution of 0.1M Glycine (pH 2.5) for 45 minutes, neutralized in 400 ml 0.1M Tris-HCl (pH 8) for 45 minutes, and blocked for 2 hours in 200 ml of 5% (w/v) non-fat dried milk in TBST. For STATs 1 and 2 immunoblots,

membranes were incubated in a 5% (w/v) solution of non-fat dried milk in TBS for 2 hours in 10 ml of a 1:1000 dilution each of anti-STAT-1 and anti-STAT-2 polyclonal antibodies, quickly rinsed twice, washed once for 15 minutes and 2 times for 5 minutes in TBST, incubated for 1h in a 1:8000 dilution (10ml total) of horse radish peroxidase (HRP)-linked anti-rabbit IgG antibody (part of the ECL kit) in TBST containing 5% (w/v) non-fat dried milk, quickly rinsed twice, washed once for 15 minutes and four times for 5 minutes with TBST. STAT proteins on membranes were detected with an ECL kit with exposures of 30 seconds.

Densitometry. Enhanced chemiluminescence exposures were analyzed by densitometry as described in Chapter 3.

Experiment 2

Details of the methodology used for estrous cycles synchronization, surgeries and infusions are described in Arnold et al. (1999).

Estrous cycle synchronization. Non-lactating dairy cows housed at the Dairy Research Unit (Hague, FL) received an injection of 25 mg of $PGF_{2\alpha}$ (Lutalyse). Estrus were detected as described for experiment 1, and was designated experimental day 0.

Surgery. On experimental day 10, cows were transported to a grassy pen at the College of Veterinary Medicine (University of Florida, Gainesville). On day 11, cows were examined by rectal palpation to confirm presence of healthy corpora lutea (CL). Briefly, after epidural and local anesthesia, uterine horns

were exteriorized through flank incisions and catheterized. Catheters were exteriorized through an orifice in the left flank, filled with 20 mM Tris-HCl, pH 8.0 containing 1 mM EDTA (750 µl) mixed with antibiotic (Nacxel®, 450 µl of a 50 mg/ml solution) and, placed into bags with iodine soaked gauze. After surgery, animals were returned to their pen.

Infusions. Either BSA (1.9 mg in 1.2 ml 20 mM Tris-HCl, 1 mM EDTA, pH 8.0) or IFN-τ (0.2 mg +1.7 mg BSA in 1.2 ml 20 mM Tris-HCl, 1 mM EDTA, pH 8.0) were infused every 12 hours from experimental days 14 to 17.

Slaughter and Tissue Collection. On the morning of experimental day 17, after the bIFN-τ infusion, animals were slaughtered by jugular exanguination and reproductive tracts were immediately removed and transported to the laboratory. Uterine lumen from the horn contralateral to CL was exposed and endometrial tissue was dissected as described for Experiment 1.

Cell extract. A technique described for ovine endometrium (Charpigny et al., 1998) was used to obtain endometrial epithelial cells for whole cell extracts. Briefly, endometrial lumen was irrigated with Hank's balanced salt solution and gently scraped with a scalpel blade. Cells were placed in a 15 ml conical tube containing Earle's medium supplemented as described above. After whole lumen had been scraped, cell suspension was centrifuged for 5 minutes (in a Dynac non-refrigerated bench centrifuge, Parsippany, NJ) at 500 x g, resuspended in 5 ml of Earle's medium and examined for viability using the trypan blue exclusion method. The cell suspension was centrifuged again,

medium was discarded and cells were resuspended in 500 μl of whole cell extract (WCE) buffer, and WCE were obtained as described in Chapter 5.

Immunoblotting. To evaluate the nature of cells scraped from endometrium (described above), 20 μg of WCE from each cow were loaded onto duplicate 7.5% acrylamide gels, submitted to SDS-PAGE and electrophoretic transfer of proteins to nitrocellulose membranes as described for Experiment 1. Membranes were probed separately for cytokeratin (1:2000 dilution) and vimentin (1:2000 dilution) as described in Chapter 5. Proteins were detected using ECL.

Volumes of WCE from each cow corresponding to 25 μg of protein were loaded onto 7.5% acrylamide gels, submitted to SDS-PAGE and electrophoretic transfer of proteins to nitrocellulose membranes as described for Experiment 1. Membranes were separately probed with antibodies against COX-2 and PLA_2 as described in Chapter 7. Proteins were detected by ECL and analyzed by densitometry.

Use of densitometry to evaluate abundance of protein signals obtained after chemiluminescence were validated. Serial dilutions of WCE positive for COX-2 were loaded onto a 7.5% acrylamide gel and submitted to electrophoresis and immunoblotting for COX-2. The ECL exposure was analyzed by densitometry.

Statistical Analysis

Variables analyzed by densitometry also were analyzed by least squares analysis of variance, using the GLM procedure of SAS (SAS, 1988). In Experiment 1, independent variables were cow, gel, treatment, cow by gel, cow by treatment and gel by treatment. In Experiment 2, independent variables were gel, treatment and gel by treatment.

Results

Experiment 1

Synthesis of secretory proteins from endometrial explants. Visual inspection of fluorographs suggested presence of two proteins of 19 (P19) and 12 kD (P12) that were regulated by bIFN-τ (Figure 4-1, panel a). Treatment with bIFN-τ enhanced synthesis and secretion of both proteins qualitatively, but densitometric analysis indicated only a numeric difference in the intensity of bands between treatments, both for P19 ($p<0.26$) and for P12 ($p<0.16$; Figure 4-1, panel b). Although the effect of cow was not significant for either P19 or P12 ($p>0.1$), cow 5497 had a numerically reduced response to treatment with bIFN-τ for both proteins. Data were re-analyzed without cow 5497 and statistical significance for treatment changed slightly ($p<0.24$) for P19 but significantly ($p<0.05$) for P12.

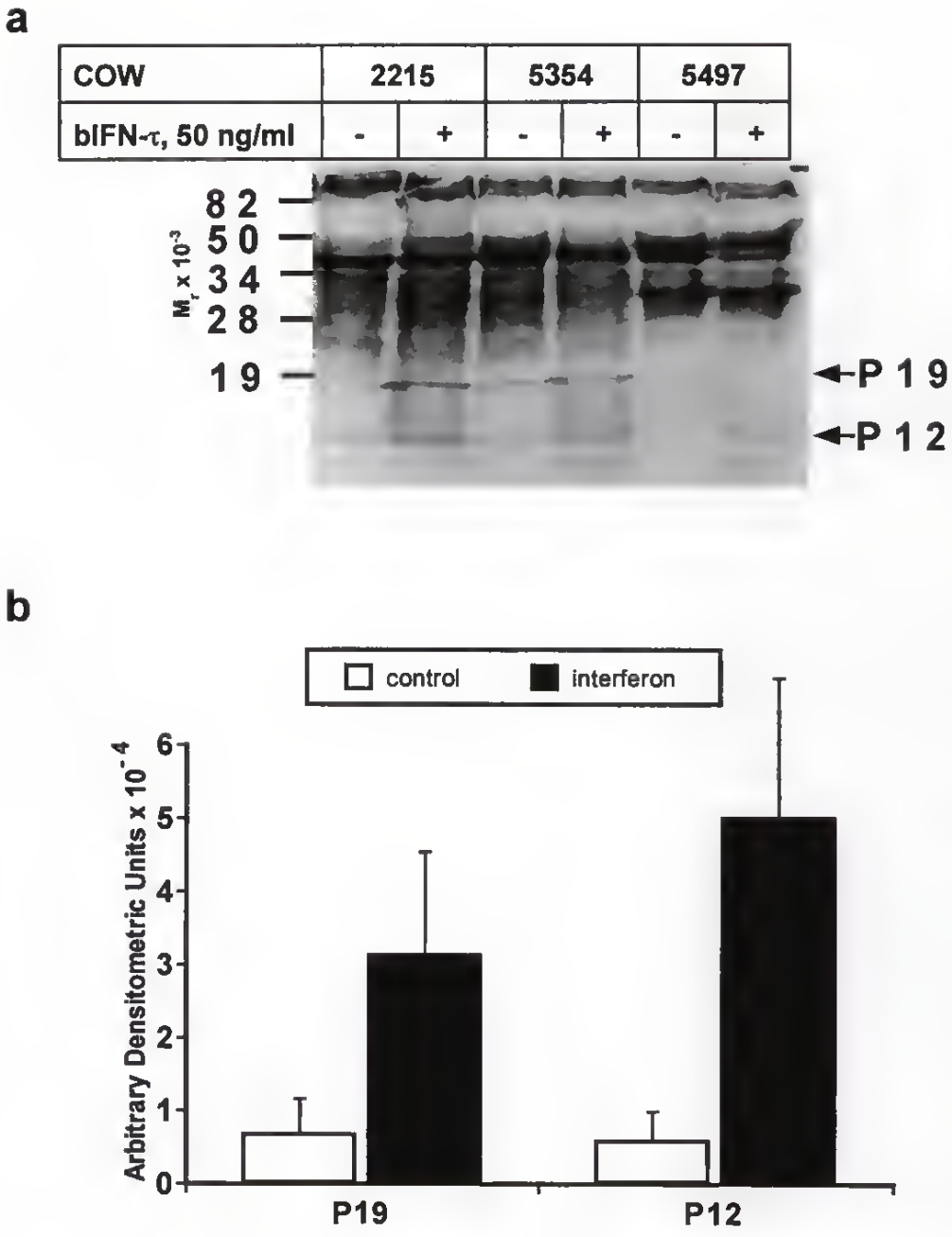


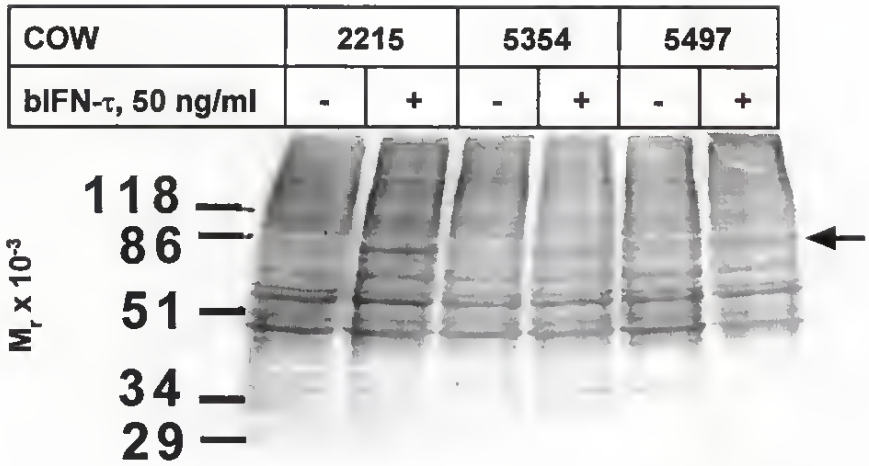
Figure 4-1. Fluorographic analysis of **newly synthesized and secreted proteins** from endometrial explants incubated for 24 h in presence (+) or absence (-) of bIFN-τ.

a) Representative fluorograph of one dimensional SDS-PAGE analysis of secretory proteins (arrows indicate differentially expressed proteins P19 and P12; b) Densitometric analysis of abundance of P19 and P12 (least squares means ±SEM).

Synthesis of intracellular proteins from endometrial explants. Visual inspection of fluorographs suggested presence of a 75 kD protein (P75) regulated by bIFN-τ (Figure 4-2, panel a). Treatment with bIFN-τ enhanced synthesis and secretion of P75 qualitatively, but densitometric analysis indicated only a numeric difference in the intensity of bands between treatments ($p<0.25$; Figure 4-2, panel b). There was a significant effect of cow ($p<0.05$). Cow 2215 had higher synthesis of P75 compared to the other cows.

2 hour cultures. Treatment of endometrial explants with bIFN-τ stimulated tyrosine phosphorylation of both STAT-1 and STAT-2 (Figure 4-3, panel a), despite a similar abundance of both proteins over time (Figure 4-3, panel b). Phosphorylation increased after 10 minutes of exposure to bIFN-τ and reached a maximum after 30 minutes. Then, phosphorylation apparently decreased by 60 minutes to increase again after 120 minutes. Changes in phosphorylation occurred despite a similar abundance of STATs 1 and 2 for all treatmentintervals (except for a reduced abundance of STAT-2 at the 0 minute sample; Figure 4-3, panel b). Overall, it was difficult to detect tyrosine phosphorylation in extracts from explants. There was consistently a high background on ECL exposures and intensity of bands was always low. Data from only one cow is presented because it was not possible to detect phosphorylation in response to bIFN-τ for the other cows.

a



b

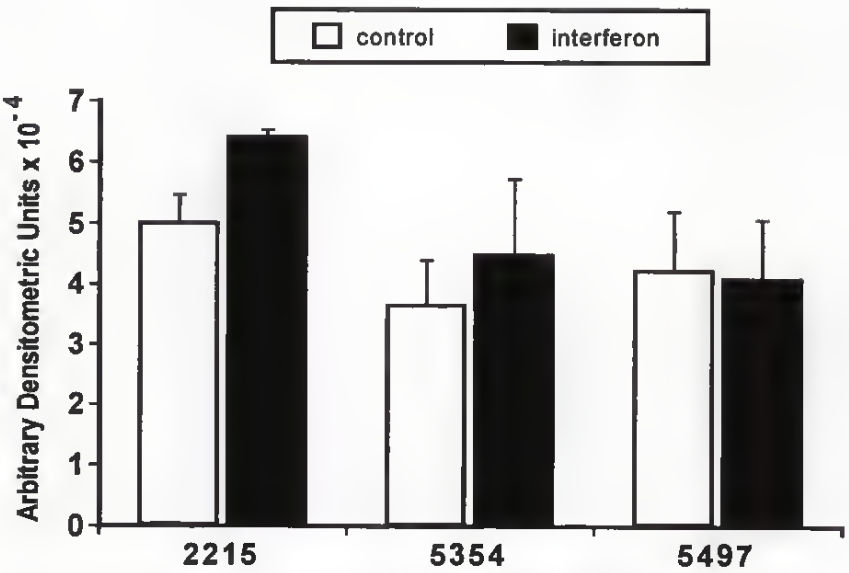


Figure 4-2. Fluorographic analysis of **newly synthesized intracellular proteins** from endometrial explants of cows 2215, 5354 and 5497, incubated for 24 h in presence (+) or absence (-) of bIFN-τ.

a) Representative fluorograph of one dimensional SDS-PAGE analysis of intracellular proteins (arrow indicates differentially expressed 75 kD protein (P75); b) Densitometric analysis of treatment by cow interaction of abundance of P75 (least squares means±SEM).

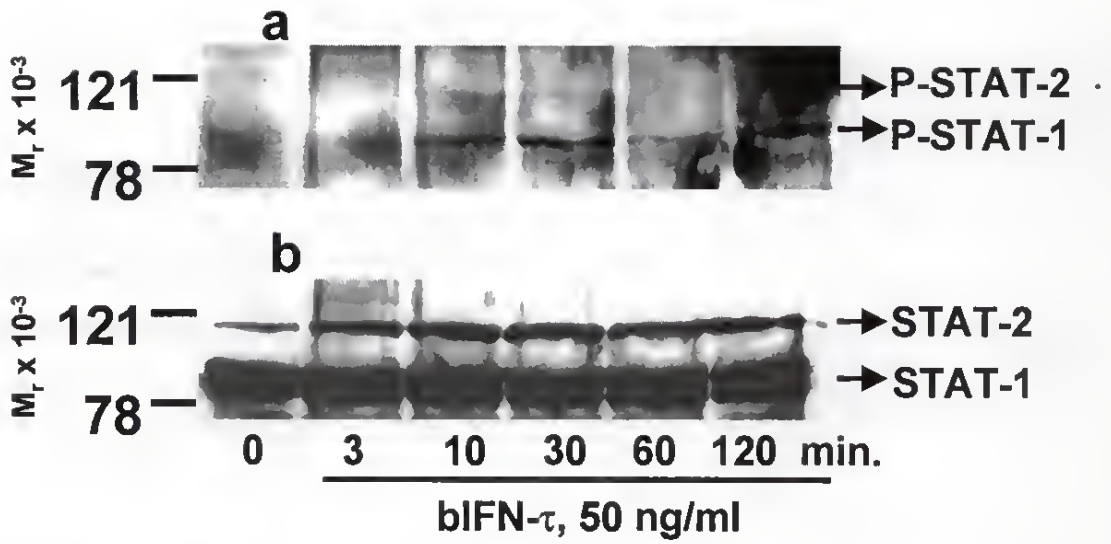


Figure 4-3. Immunoblotting analysis of STATs 1 and 2 immunoprecipitated from whole tissue extracts from endometrial explants treated with bIFN-τ for increasing intervals of time.
a) Representative enhanced chemiluminescence (ECL) exposure of **tyrosine phosphorylation of STATs 1 and 2**; b) Representative ECL exposure of **abundance of STATs 1 and 2.**

Experiment 2

Validation of densitometric technique. There was a linear decrease in arbitrary densitometric units associated with increased dilution of WCE for all exposure times tested (Figure 4-4).

Immunoblotting for cytokeratin and vimentin. Although we originally intended to use scraped endometrial luminal epithelial cells for culture, analysis of scraped cells using trypan exclusion revealed that 99% of cells were dead at the end of the scraping procedure. Therefore, these cells were used only for preparation of extracts. Regardless of in vivo treatment (i.e., BSA or bIFN-τ),

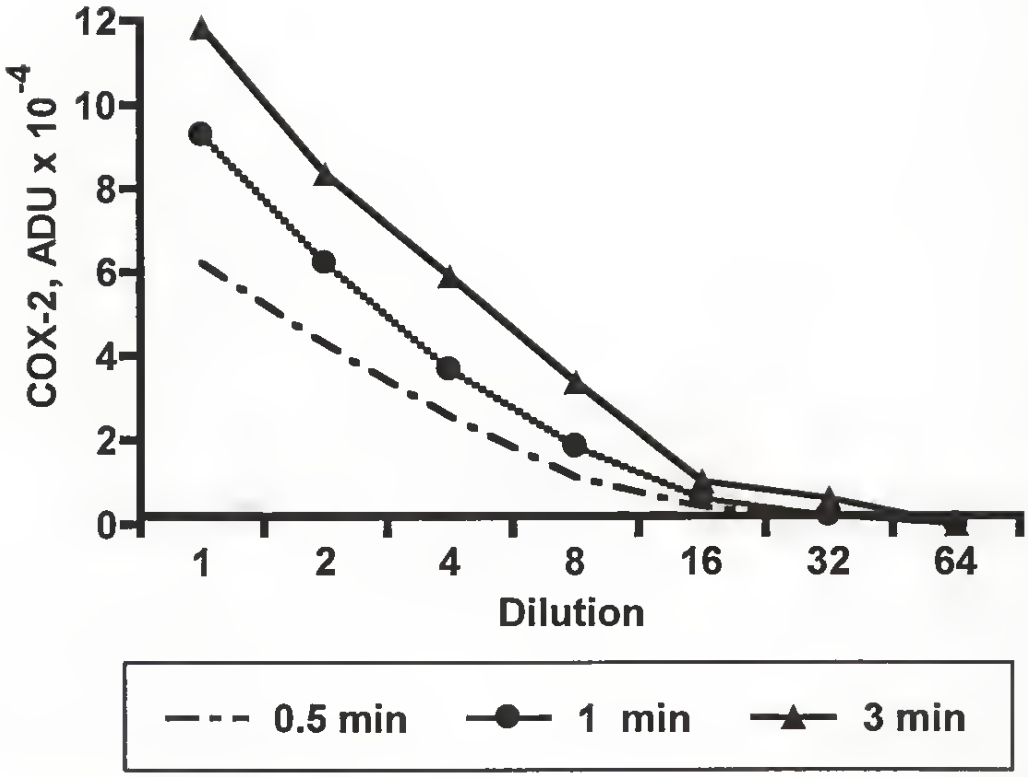


Figure 4-4. Validation of densitometry. Densitometric values of COX-2 abundance in serially diluted samples. Values were obtained after enhanced chemiluminescence exposures of 0.5, 1 and 3 minutes.

WCE from scraped cells from all cows showed a strong staining for cytokeratin (Figure 4-5). In contrast, staining for vimentin was absent for all BSA-treated cows while two bIFN-τ-treated cow showed staining for vimentin (Figure 4-6).

Immunoblotting for COX-2 and PLA_2. Abundances of COX-2 (Figure 4-7) and PLA_2 (Figure 4-8) in WCE from scraped endometrial cells were similar for cows treated with either BSA or bIFN-τ in vivo.

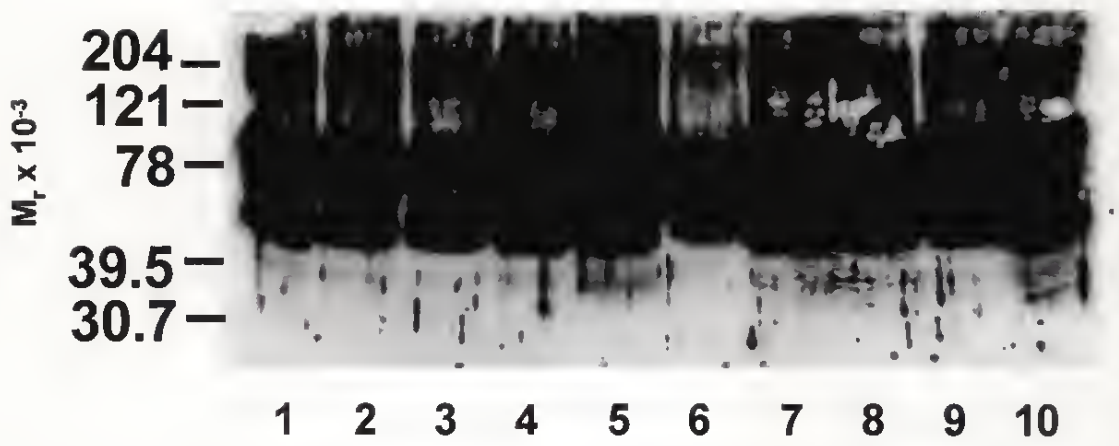


Figure 4-5. Immunoblotting analysis of **cytokeratin** in whole cell extracts from endometrial epithelial cells from cows treated in vivo with placebo or bIFN-τ. Representative enhanced chemiluminescence (ECL) exposure of abundance of cytokeratin for control cows (lines 1, 3, 5, 6 and 9) and for bIFN-τ-treated cows (lines 2, 4, 7, 8, and 10).

Discussion

Results from Experiment 1 indicated that treatment with bIFN-τ stimulated synthesis of both secretory and intracellular proteins from bovine endometrium. Moreover, the JAK-STAT pathway of signal transduction is present and operational in bovine uterine tissue, although use of explants does not provide an ideal model to study bIFN-τ-mediated signal-transduction in the endometrium.

Differential expression of at least two secretory proteins (P19 and P12) suggests that the recombinant bIFN-τ used in the present experiments is biologically active and stimulates synthesis and secretion of unique endometrial proteins. These findings agree with those reported by Naivar and coworkers (1995). Those authors identified three secretory proteins which were stimulated

Figure 4-6. Immunoblotting analysis of **vimentin** in whole cell extracts from endometrial epithelial cells from cows treated in vivo with placebo or bIFN-τ. Representative enhanced chemiluminescence (ECL) exposure of abundance of vimentin for control cows (lines 1, 3, 5, 6 and 9) and for bIFN-τ-treated cows (lines 2, 4, 7, 8, and 10).

by treatment of endometrial explants in vitro with bIFN-τ, of molecular weights 8 (P8), 16 (P16) and 28 kD (P28). Although the calculated molecular weight of the proteins found in our study were 12 and 19 kD, it is likely that they correspond to P8 and P16 in their study. The lowest (i.e., fastest migrating) molecular weight standard marker used in the electrophoresis procedure of the present experiment is 19 kD. Sizes of proteins with lower molecular weights were calculated based on a regression equation that might have slightly overestimated the calculated molecular weights of P19 and P12. Protein P8 was identified as bovine granulocyte chemotactic protein-2, a member of the alpha chemokine family (Teixeira et al., 1997). Chemokines are potent chemo-attractants for cells of the immune system and have been implicated in cell adhesion, inflammatory and angiogenic processes (Oppenheim et al., 1991). In support of a possible angiogenic role of the chemokine, Arnold and coworkers (1999) observed

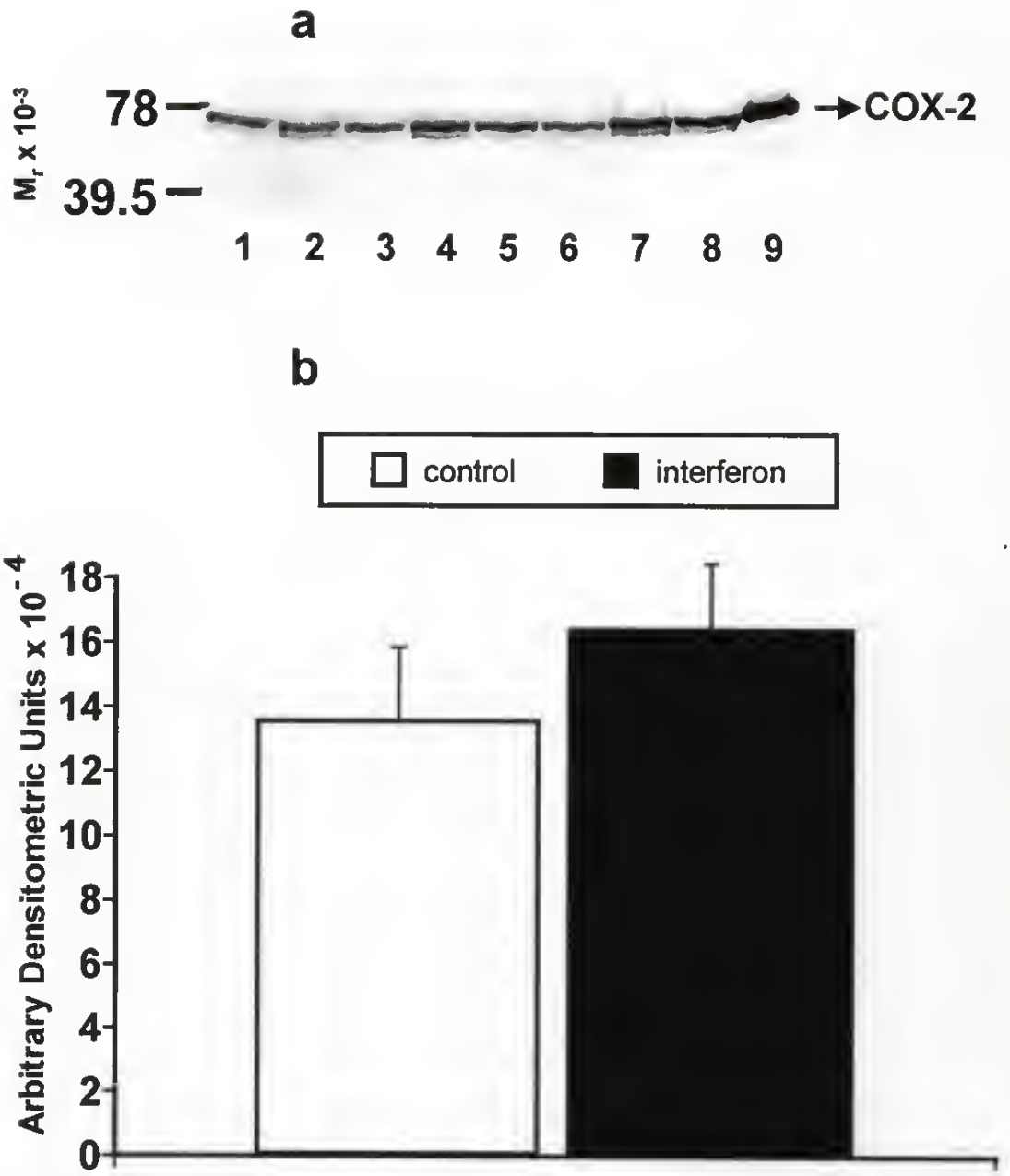


Figure 4-7. Immunoblotting analysis of **COX-2** in whole cell extracts from endometrial epithelial cells from cows treated in vivo with placebo or bIFN-τ.

a) Representative enhanced chemiluminescence (ECL) exposure of abundance of COX-2 for control cows (lines 1 to 4) and for bIFN-τ-treated cows (lines 5 to 8); line 9 is a positive control for COX-2; b) Densitometric analysis of abundance of COX-2.

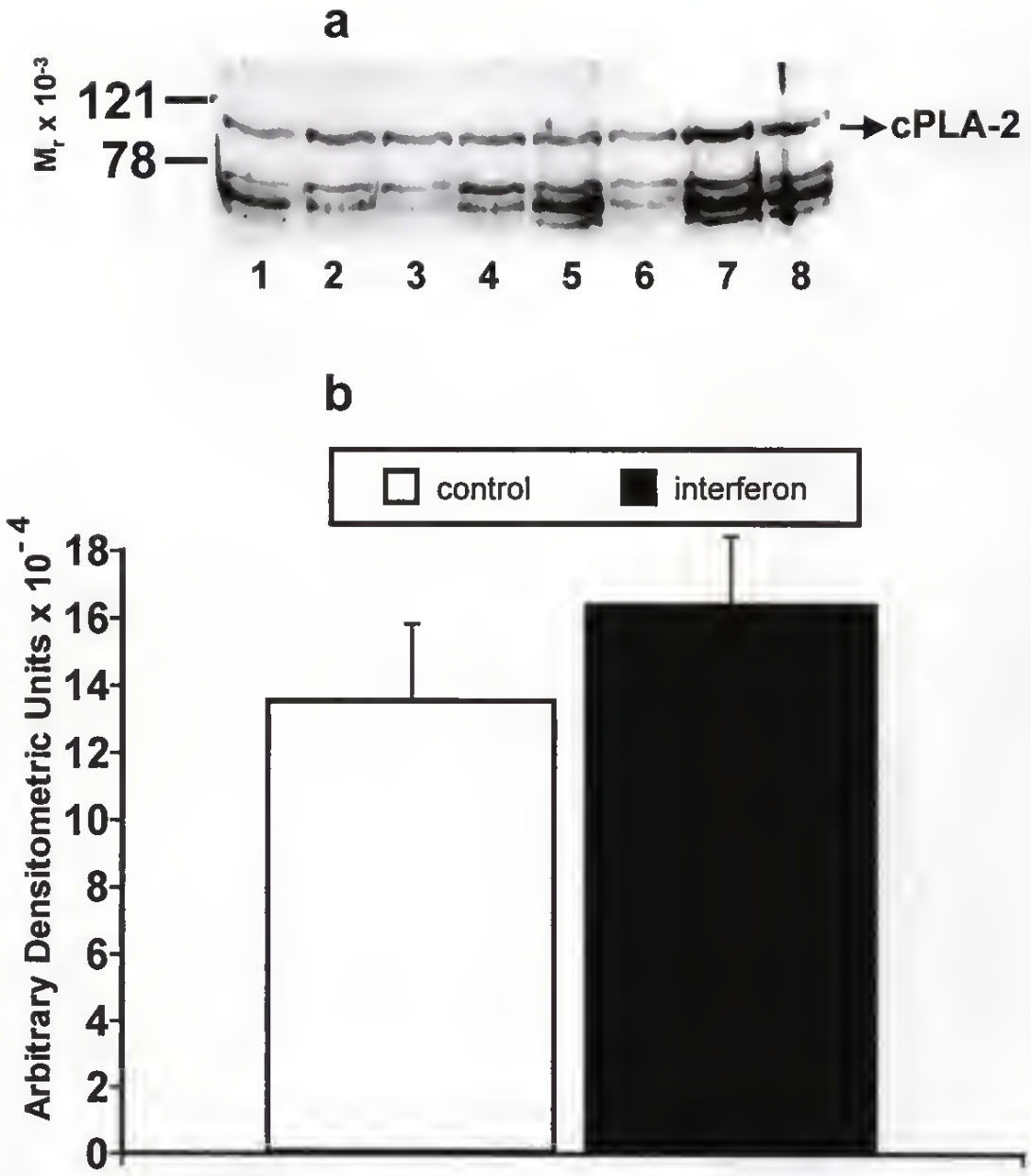


Figure 4-8. Immunoblotting analysis of PLA_2 in whole cell extracts from endometrial epithelial cells from cows treated in vivo with placebo or bIFN-τ.

a) Representative enhanced chemiluminescence (ECL) exposure of abundance of PLA_2 for control cows (lines 1 to 4) and for bIFN-τ-treated cows (lines 5 to 8); b) Densitometric analysis of abundance of PLA_2.

presence of a region of pronounced growth of capillaries in the endometrium of cows which received intrauterine infusions of bIFN-τ. Chemokines may recruit specific populations of lymphocytes to the endometrium, which could act to secrete cytokines beneficial to development of the conceptus (for reviews see Hansen, 1997; Robertson et al., 1994). Protein P16 was identified as bovine ubiquitin-cross-reactive protein (bUCRP; Austin et al., 1996b). Ubiquitin conjugates with proteins to modulate their cellular activities and also to target them to proteasomal degradation (Finley and Chau, 1991). The bUCRP cross-reacts with antibodies against ubiquitin in immunoblots (Austin et al., 1996b) and sequence analysis revealed that bUCRP maintains critical domains and residues involved in conjugation with other proteins (Hansen et al., 1999). Moreover, Johnson and coworkers (1998a) demonstrated the ability of bUCRP to form conjugates with an array of cytosolic endometrial proteins following treatment with bIFN-τ. It is possible that bUCRP plays a role on the antiluteolytic actions of bIFN-τ in the endometrium, perhaps targeting proteins involved in the pathway of $PGF_{2\alpha}$ production to alter their cellular activity and ultimately decrease secretion of $PGF_{2\alpha}$.

Examination of proteins in whole tissue extracts indicated presence of a 75 kD protein (P75) induced by bIFN-τ in endometrium. Review of the literature on interferon-induced proteins revealed presence of a ovine IFN-τ-induced protein of 70 to 80 kD (Pavlovic et al., 1993) in the endometrium of sheep, designated Mx protein (Charleston and Stewart, 1993; Ott et al., 1998). Mx

proteins are functional GTPases that are strongly induced by type I interferons in response to viral infections (Pavlovic et al., 1993). Ott and coworkers (1998) showed a presence of Mx mRNA in endometrium of day 13 to day 19 pregnant sheep, which coincide with maximum expression of IFN-τ by ovine conceptuses (Roberts et al., 1992). However, function of Mx protein in the endometrium remains elusive.

Visual evaluation of fluorographs of both secretory and intracellular proteins indicate that there is a differential responsiveness for different cows, in regard to proteins induced by bIFN-τ. Cow 2215 appears to be the most, while cow 5497 the least responsive to bIFN-τ. This phenomenon of non-uniform responsiveness to bIFN-τ was also reported by Helmer and others (1989b). They measured concentrations of plasma progesterone from cows that received intra-uterine infusions of either bovine serum albumin or bIFN-τ, as a measure of CL lifespan. Although the overall lifespan of CLs was greater for bIFN-τ-treated compared to BSA-treated-cows (26 vs. 19.5 days, respectively), CL lifespan for individual cows receiving bIFN-τ varied from 21 to 28 days. Thatcher and coauthors (1997) estimated that around 20% of cows did not respond to bIFN-τ in different experiments. This variability may be associated with embryo losses observed during the period of maternal recognition of pregnancy in cattle. Possible causes are lack of functional bIFN-τ receptors or failure of bIFN-τ to

induce appropriate intracellular signals within the endometrium, which could result in a breakdown on the antiluteolytic mechanism.

Preliminary experiments reported by Binelli and others (1996) indicated that STAT-1 protein is present and can be stimulated in endometrial explants treated with bIFN-τ in vitro. Data in the present report confirm and expand those early observations. Tyrosine phosphorylation of STATs 1 and 2 occurred in a time-dependent fashion, which was only noted in explants treated with bIFN-τ. This is in agreement with data from Silvennoinen and coworkers (1993), which also showed induction of STAT phosphorylation to be time-responsive. Signal transduction systems working through tyrosine phosphorylation are regulated acutely, to provide specific responses to specific stimuli. Therefore it is not surprising that a similar dynamic is also valid for bIFN-τ-stimulated signal transduction in the endometrium. There was no clear pattern of rise and fall of phosphorylation of STATs over time. There was an increase to a maximum level at 30 minutes, followed by a decrease by 60 and another increase at 120 minutes. This might reflect cycling dynamics of tyrosine phosphorylation of STATs.

In general, it was extremely challenging to obtain consistent results of tyrosine phosphorylation using this model of in vitro incubations of explants. Explants were chosen initially because it was considered desirable to maintain structural integrity of the endometrium and stroma-epithelium interactions to best mimic the in vivo system. Most production of $PGF_{2\alpha}$ in the endometrium comes

from epithelial cells (Danet-Desnoyers et al., 1994). Therefore, it is reasonable to assume that bIFN-τ exerts most of its regulation on epithelial cells. However, in WCE used for immunoprecipitation, proteins originated from epithelial cells were diluted with proteins coming from the stroma, capillaries etc. It is possible that STAT proteins are present in all cell types in the endometrium, but they might become tyrosine phosphorylated preferentially in the epithelium. Due to cellular heterogeneity of explants, immunoprecipitation of WCE collected a pool of STATs from different cell types, and epithelial, tyrosine-phosphorylated STATs are in too small of a proportion to be detected clearly. This would explain why tyrosine phosphorylation was difficult to detect regardless of the abundant presence of STATs. An alternative explanation is that tissue phosphatases quickly de-phosphorylated STATs, which then could not be detected in immunoblots. Although phosphatase inhibitors such as β–glycerophosphate and Na_3VO_4 were present in the homogenization buffer and homogenization was performed on ice, it is possible that compartmentalized pools of phosphatases originally present in the whole tissue were disrupted and an acute removal of tyrosine phosphates took place. Whatever the reason, it became clear that a different model of study would be necessary to evaluate critically the dynamics of bIFN-τ-stimulated JAK-STAT pathway in the endometrium.

Experiment 2 tested an alternative system for study of bIFN-τ actions in the endometrium, using scraped epithelial endometrial cells. Initial intention was to use scraped cells for culture, to measure phosphorylation of STAT proteins in

response to bIFN-τ in vitro. However, there was a 99% mortality rate (as measured by trypan blue exclusion), and the system was considered inadequate for such purpose. This was in contrast to Charpigny and others (1999), which were able to measure conversion of labeled arachidonic acid into several prostaglandin classes using this system with ovine endometrium. A second purpose was to compare the abundance of PLA_2 and COX-2 in cells obtained from cows previously treated with control protein (BSA) or bIFN-τ in vivo. Since PDBu-stimulated $PGF_{2\alpha}$ production was attenuated in explants from these same cows treated with bIFN-τ in vivo (Arnold et al., 1999), it was hypothesized that a lower abundance of PLA_2 and COX-2 would explain the $PGF_{2\alpha}$ results. However, similar abundance of both enzymes indicates that bIFN-τ regulation of $PGF_{2\alpha}$ is exerted through regulation of cellular activity of these enzymes.

In conclusion, the experiments reported in this Chapter indicated that bIFN-τ stimulated synthesis of endometrial proteins which may correspond to the previously described bGCP-2, bUCRP and Mx proteins. Such proteins may have specific roles on the process of maternal recognition of pregnancy, however, such roles have not been confirmed. In addition, bIFN-τ stimulates a functional JAK-STAT pathway in the endometrium. Yet, an alternative in vitro model of study will be required for further understanding of details of the bIFN-τ-induced signal transduction in the endometrium and its relationship with the antiluteolytic actions of bIFN-τ. Scraped endometrial epithelial cells proved not

to be an adequate system for such studies. However, they could be used to verify steady state levels of endometrial epithelial proteins. The newly developed BEND cell line (Austin et al., 1996b) may prove to be a useful model to conduct studies in the signal transduction system employed by bIFN-τ in the endometrium.

CHAPTER 5
BOVINE INTERFERON-TAU STIMULATES THE JAK-STAT PATHWAY IN BOVINE ENDOMETRIAL EPITHELIAL CELLS

Introduction

In cattle, maintenance of pregnancy is accomplished by suppression of luteolytic pulses of $PGF_{2\alpha}$ from the pregnant uterus. Presence of a conceptus maintains the corpus luteum (CL) at around day 17 of pregnancy (Thatcher et al., 1997). Several studies indicate that bovine (b) interferon (IFN-τ) is secreted by the elongating embryo during the time of CL maintenance (Bartol et al., 1985; Helmer et al., 1987; Farin et al., 1990). Moreover, bIFN–τ is able to suppress the oxytocin-induced release of $PGF_{2\alpha}$ in vivo (Meyer et al., 1995) and in vitro with cultured endometrial epithelial cells (Danet-Desnoyers et al., 1994; Xiao et al., 1999) and increases CL lifespan in cows that received infusions of bIFN–τ in utero (Meyer et al., 1995). In order to exert its anti-luteolytic functions, bIFN–τ must: (1) interact with endometrial epithelial cells, since they are the major source of uterine $PGF_{2\alpha}$ (Danet-Desnoyers et al., 1994) and (2) stimulate signal transduction mechanisms to change the function of endometrial epithelial cells to suppress $PGF_{2\alpha}$ pulses. Although binding of bIFN–τ has been demonstrated in bovine endometrial cells (Li and Roberts, 1994), very little work has been

conducted to study the signal transduction system activated by bIFN–τ. Initial work of our laboratory (Binelli et al., 1996) suggests that bIFN–τ activates the JAK-STAT signal transduction pathway (Darnell et al., 1994; Darnell, 1997) in bovine endometrial cells. Our hypothetical model (Figures 2-1 and 2-3) is that binding of bIFN–τ to its receptor elicits tyrosine-phosphorylation of STAT proteins in the cytosol of endometrial epithelial cells (Levy et al., 1989). Phosphorylated STATs (STAT-1, -2 and -3) form homo- and heterodimers (Schindler and Darnell, 1995; Darnell, 1997) that translocate to the nucleus where they activate transcription of bIFN–τ induced genes and protein synthesis (Schindler et al., 1992). Synthesized proteins could act to decrease synthesis of $PGF_{2\alpha}$. Binelli and coworkers (1996; Chapter 4) showed that STAT-1 is present in bovine endometrial explants and that bIFN–τ is able to induce tyrosine-phosphorylation of STAT-1. The present Chapter contains results from a series of studies designed to gain further knowledge on the signal transduction pathway stimulated by bIFN–τ in bovine endometrium. Our model of study was a recently developed line of bovine endometrial epithelial cells (BEND cells), obtained from day 14 cyclic cows (Austin et al., 1996b). Specific objectives were: (1) to test the dose responsiveness of bIFN–τ-induced phosphorylation of STAT-1, and -2, (2) to study the time responsiveness of bIFN–τ-induced phosphorylation of STAT-1, -2, and -3; (3) to verify whether bIFN–τ induces nuclear translocation of STAT proteins; and, (4) to examine the formation of STAT-STAT complexes.

Materials and Methods

Materials

Reagents and materials utilized for preparation of cell extracts, immunoprecipitation and immunoblotting are the same as described in Chapter 4, except where noted. Monoclonal anti-vimentin clone V9 antibody (catalog number V6630; 6.2 mg/ml IgG1), monoclonal anti-pan cytokeratin antibody (catalog number C2562; 45.6 mg total protein/ml), Ham's F-12 (catalog number N6760), MEM (catalog number M7395), antibiotic-antimycotic solution (AbAm; catalog number A9909), insulin (catalog number I5500), D-valine (catalog number V1255), horse serum (catalog number H1138) and trypsin solution were from Sigma Chemical Co.(St. Luis, MO). Fetal bovine serum was acquired from Atlanta Biologicals (catalog number S11550; Norcross, GA). Fifteen ml propylene tubes and 100 x 16 mm polypropylene centrifuge tube were from Fisher Scientific (Pittsburgh, PA). Tissue culture dishes were from Corning Glass Works (Corning, NY). Recombinant bIFN–τ (dissolved in 20 mM Tris-HCl, 1 mM EDTA, pH 8.0 to 200 μg/ml; 1.08 x 10^7 units of antiviral activity) was a generous gift from Dr. Michael Roberts (University of Missouri). Cell scraper and cell culture flasks (175 cm^2, polystyrene with vented cap; catalog number 83.1812.002) were from Sarstedt, Inc. (Newton, NC). Anti-STAT-1 (E-23; catalog number SC-346, 200μg/ml), anti-STAT-2 (C-20; catalog number SC-476,

200μg/ml) and anti-STAT-3 (C-20; catalog number SC-482, 200μg/ml) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-phosphotyrosine (PY-20, 1 mg/ml) antibody was from Transduction Laboratories (Lexington, KY). Enhanced Chemiluminescence kit (Renaissance® Western Blot Chemiluminescence Reagent Plus) was from NEN™ Life Science Products (Boston, MA).

Cell Culture and Cell Extracts

BEND cells (Austin et al., 1996b; Staggs et al., 1998) were grown to confluence in culture medium (40% Ham's F-12, 40% MEM, 10 ml AbAm/l, 200 U insulin/l, 0.0343g D-valine/L, 10% fetal bovine serum, 10% horse serum; complete culture medium) in cell culture flasks at 37 C in a CO_2 incubator (Sanyo, model MCO 17AI, Sanyo Electric Co., Ltd, Japan) under a humidified atmosphere containing 95% O_2 and 5% CO_2. Upon reaching confluence cells were collected from culture flasks and were used (1) for experiments, (2) plated on new culture flasks to maintain a stock of growing cells for subsequent experiments and/or (3) frozen for maintaining a frozen stock. To collect cells from culture flasks, medium was discarded and 10 ml of trypsin solution were added to the flask and placed in the incubator for 5 minutes. Gentle tapping of corners of the flask was used to dislodged cells. Five ml of complete culture medium were added to the flask to stop trypsin reaction, and all contents from the flask were transferred to a 15 ml conical polypropylene tube. Cells were

centrifuged in a Dynac non-refrigerated bench centrifuge (Parsippany, NJ) with the dial "speed" set to 70 (approximately 700xg) for 5 minutes. Supernatant was discarded and cells were resuspended in 5 to 10 ml of medium. About one fifth of this suspension (~1 x 10^6 cells; determined by hematocytometer count) was transferred to a fresh culture flask containing 50 ml of complete culture medium to maintain a stock of growing cells for subsequent experiments. Remaining cell suspension was divided according to the number of plates needed in a particular experiment. For freezing, cells were diluted to 4 x 10^6 cell /ml of complete culture medium. Then to 500 µl of that suspension, 500 µl of freezing medium (20% DMSO in complete culture medium) were added dropwise and transferred to a cryovial. Cryovials were stored at -20 C for 2 hours, transferred to -80 C overnight and finally stored in a liquid nitrogen tank. Cell morphology was observed in each passage using a Nikon (model TMS) inverted microscope, and photographed using an Olympus inverted microscope (model CK2, Japan) equipped with a closed circuit TV camera (model DCE-107; Sony Corp., Japan) and connected to a color video copy processor (model CP110U; Mitsubishi Corp., Japan). Cells were obtained from repeated passages until cells started to change morphology (see Results) and growth rate decreased. When decreased growth rate was noticed, cells from a frozen stock were thawed (quickly, submerging cryovial in a 37C water bath), plated (50 ml complete culture medium) and upon reaching confluence, were used as a stock.

Freshly harvested BEND cells (~1x10^6 cells)were platted in 150x25 mm sterile, polystyrene, tissue culture dishes in 40 ml of complete culture medium. Plates were incubated at 37 C and experiments were conducted when cells reached 90% confluency as determined by microscopic evaluation of plates. For a given experiment, cells were washed (20 ml) and incubated (19 ml) in serum free medium for 45 minutes. Recombinant bIFN–τ treatments were diluted in 1 ml of serum-free medium and were added as described below for specific experiments. To end bIFN–τ incubations, plates were quickly transported to a walk in cooler (4 C), where all subsequent steps of extract preparation were conducted. Culture medium was discarded and cells were rinsed twice in ice-cold PBS containing 1 mM Na_3VO_4 and 5 mM NaF. As indicated below, different experiments utilized either whole cell extracts (WCE; Figure 5-1) or cytosolic (CE) and nuclear extracts (NE; Figure 5-2). Therefore, slight differences in procedures were necessary, as indicated below. Cells were washed briefly in 1 ml of appropriate ice-cold extraction buffer [WCE buffer: 50 mM Tris, pH 8.0, 300 mM NaCl, 20 mM NaF, 1 mM $Na_3 VO_4$, 1 mM $Na_4P_2O_7$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10% v/v Glycerol, 0.5% v/v NP-40 and 10 μg/ml each aprotinin, leupeptin and pepstatin (Dr. Douglas Leaman, personal communication) or hypotonic buffer: 12 mM Hepes pH 7.9, 4 mM Tris pH 7.9, .6mM EDTA, 10 mM KCl, 5 mM $MgCl_2$, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM NaF, 0.5 mM PMSF, 0.6 mM DTT and 10 μg/ml each aprotinin, leupeptin, pepstatin (Ghislain and Fish, 1996) for CE and NE] and allowed to drip dry.

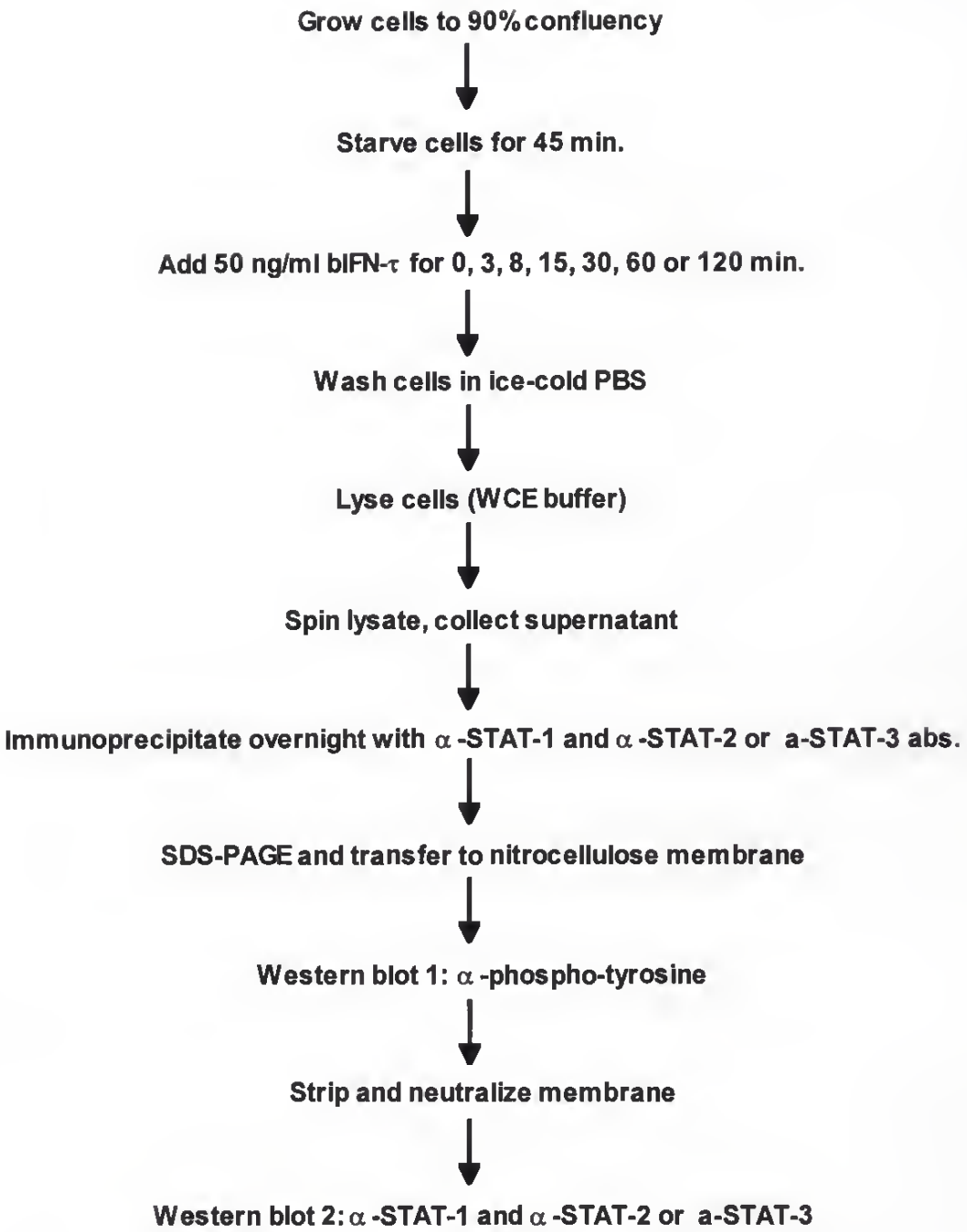

Grow cells to 90% confluency
Starve cells for 45 min.
Add 50 ng/ml bIFN-τ for 0, 3, 8, 15, 30, 60 or 120 min.
Wash cells in ice-cold PBS
Lyse cells (WCE buffer)
Spin lysate, collect supernatant
Immunoprecipitate overnight with α -STAT-1 and α -STAT-2 or a-STAT-3 abs.
SDS-PAGE and transfer to nitrocellulose membrane
Western blot 1: α -phospho-tyrosine
Strip and neutralize membrane
Western blot 2: α -STAT-1 and α -STAT-2 or a-STAT-3

Figure 5-1. Outline of experimental sequence involving **immunoprecipitation of whole cell extracts** of BEND cells treated with bIFN-τ for increasing intervals of time. See text for details.

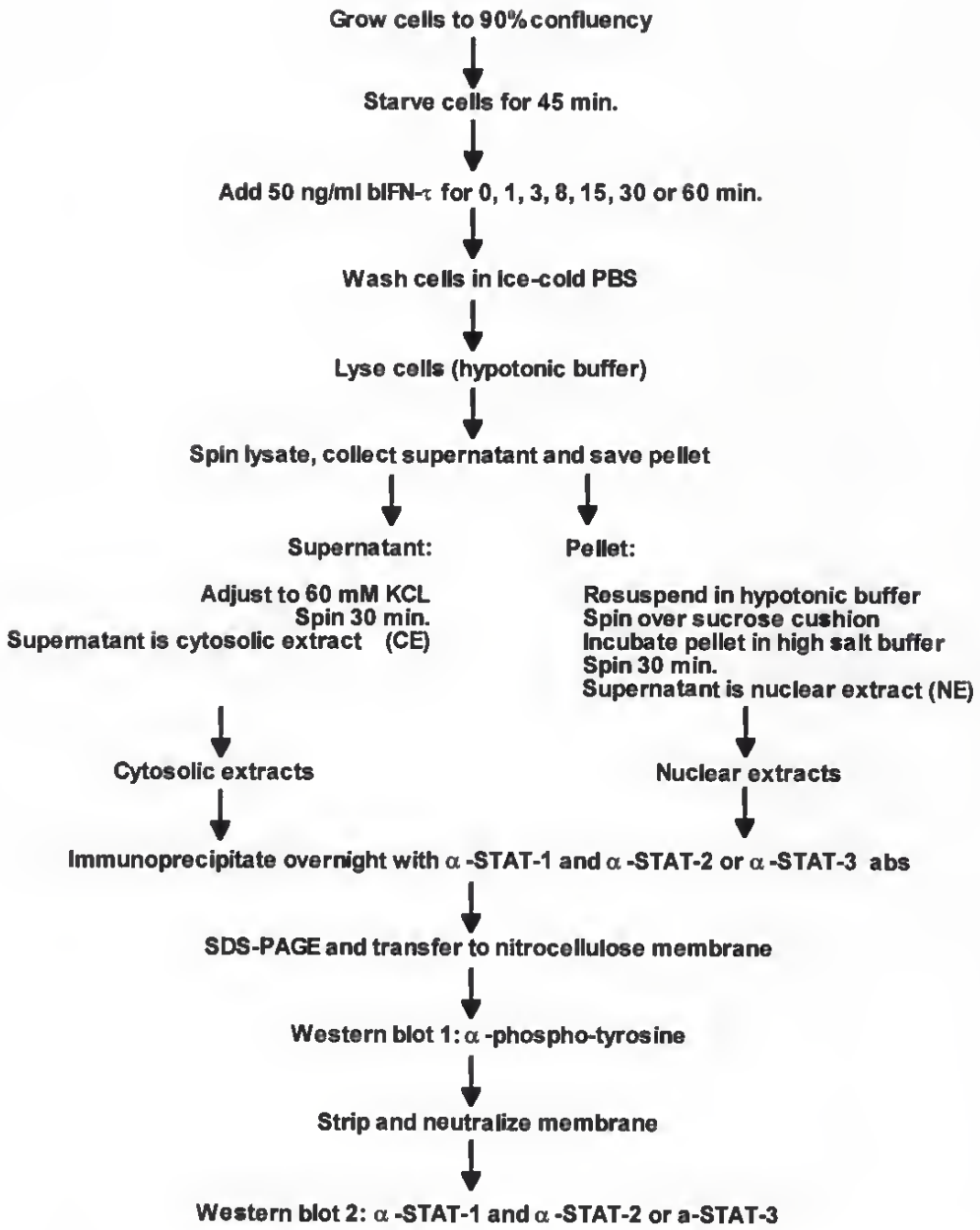

Grow cells to 90% confluency
Starve cells for 45 min.
Add 50 ng/ml bIFN-τ for 0, 1, 3, 8, 15, 30 or 60 min.
Wash cells in ice-cold PBS
Lyse cells (hypotonic buffer)
Spin lysate, collect supernatant and save pellet
Supernatant:
Adjust to 60 mM KCL
Spin 30 min.
Supernatant is cytosolic extract (CE)
Pellet:
Resuspend in hypotonic buffer
Spin over sucrose cushion
Incubate pellet in high salt buffer
Spin 30 min.
Supernatant is nuclear extract (NE)
Cytosolic extracts
Nuclear extracts
Immunoprecipitate overnight with α -STAT-1 and α -STAT-2 or α -STAT-3 abs
SDS-PAGE and transfer to nitrocellulose membrane
Western blot 1: α -phospho-tyrosine
Strip and neutralize membrane
Western blot 2: α -STAT-1 and α -STAT-2 or a-STAT-3

Figure 5-2. Outline of experimental sequence involving **immunoprecipitation of cytosolic and nuclear extracts** of BEND cells treated with bIFN-τ for increasing intervals of time. See text for details.

Cells were scraped from plates in the presence of 1 ml of appropriate extraction buffer, transferred to a microcentrifuge tube and incubated on ice for at least 10 minutes. Subsequently, cells were aspirated back and forth 5 times through a 25 gauge needle attached to a tuberculin syringe to complete lysis. To obtain WCE, lysates were spun in a microcentrifuge (model 235b, Fisher Scientific, Pittsburgh, PA; 13000 x g) for 2 minutes and supernatants were used for immunoprecipitation. To obtain CE and NE (Ghislain and Fish, 1996), lysates were spun in a microcentrifuge (13000 x g) for 20 seconds. Appropriate volume of 1M KCl was added to supernatants (cytosolic fractions) to adjust final concentration to 60 mM KCl. Then supernatants were spun for 30 minutes in a microcentrifuge (13000 x g) and used for immunoprecipitation. The pellet (from initial 20 seconds spin; crude nuclei) was resuspended in 1 ml hypotonic buffer, layered over 9 ml of a 30% sucrose solution of hypotonic buffer in a 100 x 16 mm polypropylene centrifuge tube and centrifuged at 7000 x g for 8 minutes at 4 C in a refrigerated Sorvall centrifuge (model RC-3B, equipped with a H-6000A rotor, Du Pont Co., Wilmington, DE). The pellet (nuclear fraction) was resuspended and incubated in a rotating device ("Roto-torque", heavy duty rotator, Cole Parmer, Chicago, IL; lever setting at "low", speed setting at 7) with 150 μl high salt buffer (hypotonic buffer containing 300mM KCl and 20% glycerol) for 30 minutes and clarified by centrifugation (20 minutes, 13000 x g). Resulting supernatant (NE) was diluted to1 ml with hypotonic buffer, adjusted to 60 mM KCl with appropriate volume of 1 M KCl, and used for immunoprecipitation.

Immunoprecipitation

Immunoprecipitations were conducted as described in Chapter 4, except when noted. Briefly, protein-A agarose beads were prepared as described in Chapter 4 and incubated with anti- STAT-1, -STAT-2 and / or -STAT-3 antibodies (1 μg each/sample) overnight. Beads were then washed thrice in WCE or hypotonic buffer, as appropriate, resuspended in the same buffer and added (50 μl of slurry/sample) to WCE, CE or NE as appropriate. Samples were immunoprecipitated overnight at 4 C, washed thrice in 1 ml of the appropriate buffer and once with 62.5 mM Tris-HCl pH 6.8. Proteins were solubilized by incubating beads for 5 minutes at 100 C with reducing Laemmli buffer. Samples were pulse-spun in microcentrifuge and supernatants used for immunoblots.

Immunoblots

Immunoblotting was conducted as described in Chapter 4. Briefly, immunoprecipitated proteins were separated in 7.5% acrylamide gels by 1-dimensional SDS-PAGE (150 V, 100 mA for 50 minutes). Gels were incubated in transfer buffer and proteins were transferred to nitrocellulose membranes using a mini-gel transfer tank apparatus (350 V, 100 mA for 60 minutes, Bio-Rad apparatus or maximum voltage, 400 mA for 4 hours, Hoffer apparatus). After transfer, membranes were blocked for 2 hours in 2% (w/v) gelatin in TBST (phosphotyrosine immunoblots) or 5% (w/v) non-fat dried milk (STAT

immunoblots). Membranes were washed for 15 minutes in TBST and incubated with the appropriate primary antibody. For phosphotyrosine immunoblots, membranes were incubated for 1 hour in a 1:1000 dilution of anti-phosphotyrosine monoclonal antibody (PY-20) in 2% gelatin in TBS, washed 4 times for 10 minutes in TBST, incubated for 1h in a 1:8000 dilution of horse radish peroxidase (HRP)-linked anti-mouse antibody diluted in TBST containing 2% gelatin, washed once for 15 minutes and four times for 5 minutes with TBST. Tyrosine-phosphorylated proteins on membranes were detected with an enhanced chemiluminescence (ECL) kit with exposures varying from 10 seconds to 10 minutes. For STATs 1, 2 and 3, membranes were incubated for 2 hours in a 1:1000 dilution each of either anti-STAT-1 and anti-STAT-2, or, anti-STAT-3 polyclonal antibodies in 5% non-fat dried milk diluted in TBS, washed once for 15 minutes and 2 times for 5 minutes in TBST, incubated for 1 hour in a 1:8000 dilution of horse radish peroxidase (HRP)-linked anti-rabbit antibody in TBST containing 5% non-fat dried milk, washed once for 15 minutes and four times for 5 minutes with TBST. STAT proteins on membranes were detected with an ECL kit, with exposures varying from 5 seconds to 5 minutes.

Nature of BEND Cells

To confirm that the BEND cells were primarily from epithelial origin, WCEs were obtained from BEND cells at the second (P2), fourth (P4), ninth (P9) and thirteenth(P13) passages. Increasing amounts of protein from extracts of each

passage were loaded in duplicate 7.5% acrylamide mini-gels. Proteins were transferred to nitrocellulose membranes, which were separately immunoblotted with monoclonal antibodies against vimentin (a marker for stromal cells; 1:2000 final dilution) and cytokeratin (a marker for epithelial cells; 1:2000 final dilution). Similar immunoblotting procedures as described above were used.

Dose Response to bIFN–τ

To test for specificity of bIFN–τ on STAT phosphorylation and in order to choose a dose to use in subsequent experiments, seven plates of BEND cells were assigned randomly to receive 0, 3.125, 6.25, 12.5, 25, 50 or 100 ng/ml b-IFN–τ for 15 minutes. In the present and subsequent experiments, bIFN-τ was diluted from a stock containing 200 μg/ml bIFN-τ. Total mass of bIFN-τ required for a given plate was calculated (e.g., 50 ng/ml x 20 ml of medium in a well = 1000 ng bIFN-τ), and the volume of stock containing the mass of bIFN-τ required was calculated [e.g., 1000 ng bIFN-τ needed/(200ng/μl) stock = 5μl stock] and mixed with medium to a final volume of 1 ml, which was added to the culture dish. Whole cell extracts were obtained and immunoprecipitated with anti-STAT-1 and -2 antibodies and analyzed by immunoblotting for anti-phosphotyrosine and for anti-STAT-1 and -2.

Time Response to bIFN–τ

Since tyrosine phosphorylation of proteins is an acutely regulated event, this experiment was designed to study the phosphorylation response of STATs to bIFN–τ overtime. Seven plates of BEND cells were assigned randomly to receive 50 ng/ml bIFN–τ for 3, 8, 15, 30, 60 or 120 minutes, or to receive nothing ("0 minutes"/control treatment). Whole cell extracts were immunoprecipitated with anti-STAT-1 and -2 antibodies and analyzed by immunoblotting for anti-phosphotyrosine and anti-STAT-1 and -2. The same experiment was repeated but WCE were immunoprecipitated with anti-STAT-3 antibody and analyzed by immunoblotting for anti-phosphotyrosine and anti-STAT-3.

Validation of Immunoprecipitation and Immunoblots Procedures

To verify the specificity of antibodies used for immunoprecipitation, WCE from untreated cells were immunoprecipitated with no antibody, normal rabbit serum, anti-STAT-1, -2 or -3 antibodies or a combination of anti-STAT-1 and -2 (1 μg each) and analyzed by immunoblotting for STATs 1, 2 or 3 as appropriate.

Specificity of anti-phosphotyrosine antibody used for immunoblots was tested in WCE of cells that had been treated with bIFN–τ (50 ng/ml for 8 minutes). Whole cell extracts were immunoprecipitated with anti-STAT-1 and -2 antibodies or anti-STAT-3 antibody (1μg each) and analyzed by immunoblotting with no antibody, normal rabbit serum or anti-phosphotyrosine antibody.

Specificities of anti-STAT antibodies used for immunoblots were tested in WCE of untreated cells. Whole cell extracts were immunoprecipitated with anti-STAT-1 and -2 antibodies or anti-STAT-3 antibody (1μg each) and analyzed by immunoblotting with no antibody, normal rabbit serum or either a combination of anti-STAT-1 and -2 or anti-STAT-3 antibodies.

Nuclear Translocation of STATs

In order to stimulate gene transcription, STATs must first be phosphorylated in the cytoplasm and then translocated to the nucleus. The aim of this experiment was to study the time responsiveness of phosphorylation of STATs to bIFN–τ in CE and NE obtained from the same cells. Plates of BEND cells were assigned randomly to receive 50 ng/ml bIFN–τ for 1,3, 8, 15, 30, 60 or 120 minutes, or to receive nothing ("0 minutes"/control treatment). Cytosolic extracts and NE from each plate were immunoprecipitated with anti-STAT-1 and -2 antibodies and analyzed by immunoblotting for anti-phosphotyrosine and anti-STAT-1 and -2. The same experiment was repeated but CEs and NEs were immunoprecipitated with anti-STAT-3 antibody and analyzed by immunoblotting for anti-phosphotyrosine and anti-STAT-3. To confirm that the time-responsiveness to bIFN–τ was specific, this experiment was repeated, except that bIFN–τ was not added at any time point, and CE and NE were

immunoprecipitated with anti-STAT-1, -2 and -3 antibodies simultaneously (1 μg each).

Coimmunoprecipitation of STATs

Association of proteins in complexes may be detected by coimmunoprecipitation methodology. To verify whether bIFN–τ induces formation of complexes of STATs 1 and 2 with STAT-3, eight plates of BEND cells were assigned randomly to be treated with 50 ng/ml of bIFN–τ for 1, 3, 8, 15, 30, 60 or 120 minutes or nothing ("0 minutes"/control treatment). Cytosolic extracts and NE were obtained from each plate and immunoprecipitated with anti-STAT-3. Immunoprecipitated proteins were analyzed by immunoblots for STAT-1 and -2, phosphotyrosine and STAT-3, in that order. In a separate experiment, designed to test whether bIFN–τ induces formation of complexes of STAT-3 with STATs 1 and 2, CE and NE were immunoprecipitated with STATs 1 and 2 and proteins were analyzed by immunoblotting for STAT-3, phosphotyrosine and STATs 1 and 2, in that sequence.

Densitometric Analysis

Abundance (i.e., amount of chemiluminescence signal associated with a given protein present in the sample and detected with an antibody specific for that protein) and phosphorylation (i.e., amount of chemiluminescence signal associated with phosphorylation of a given protein present in the sample and

detected with an antibody specific for phosphotyrosil groups of any protein) of STATs in the dose response, nuclear translocation and coimmunoprecipitation experiments were analyzed by densitometry as described for Chapter 3. Density. values for bands were adjusted for the background of each individual lane.

Statistical Analysis

Variables analyzed by densitometry were also analyzed by least squares analysis of variance, using the GLM procedure of SAS (SAS, 1988). Independent variables were dose and replicate for dose response experiments and time and replicate for time response experiments. For time response experiments, means were compared using a series of pre-planned orthogonal contrasts (0 vs. the average of 3, 8, 15, 30, 60 and 120 minutes; 3 vs. the average of 8, 15, 30, 60 and 120 minutes; 8 vs. the average of 15, 30, 60 and 120 minutes; 15 vs. the average of 30, 60 and 120 minutes; 30 vs. the average of 60 and 120 minutes; 60 vs. 120 minutes).

Results

Nature of BEND Cells

Visual evaluation of BEND cells at passage 5 suggests that cells are largely of epithelial origin, since they are mostly round in shape (Figure 5-3, panel a). A few elongated (perhaps of stromal origin) are also noted, and larger,

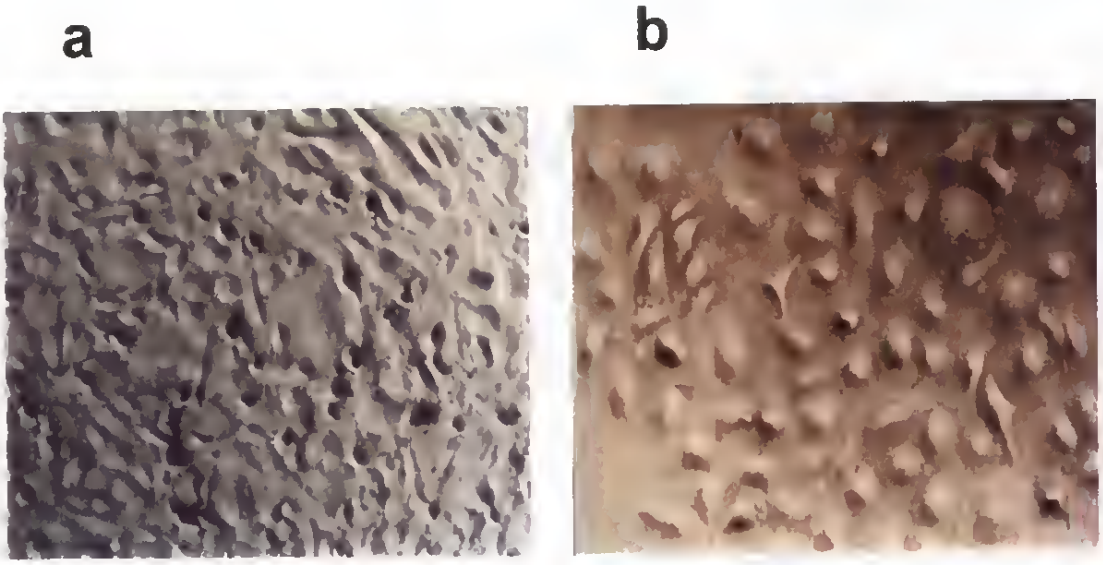


Figure 5-3. Morphology of BEND cells at different stages of growth.
a) Passage 5; b) Passage 27. Note majority of cells in "a" have round, compact morphology, typical of epithelial cells, but elongated cells with stromal morphology (top center-right) is also present in "a". Note much lower cell density and the "spread-out" phenotype of most cells in "b". Note at least one cell with same morphology in "a", on the center-right. Magnification: 100X.

round and flattened cells are also present in less number. There is a noticeable change in cell morphology when cells of a more advanced passage are examined (passage 27; Figure 5-3 panel b). Cells on P27 stopped growing and were not used further for experiments. Strong immunoreactivity for cytokeratin was noted regardless of cell passage number (Figure 5-4 panels a and b). Staining for vimentin was always less intense than for cytokeratin (Figure 5-4 panels c and d). Staining was not observed in extracts from cells of P2, but all other passages revealed presence of vimentin. No increase in vimentin intensity was observed in later passages compared to earlier passages.

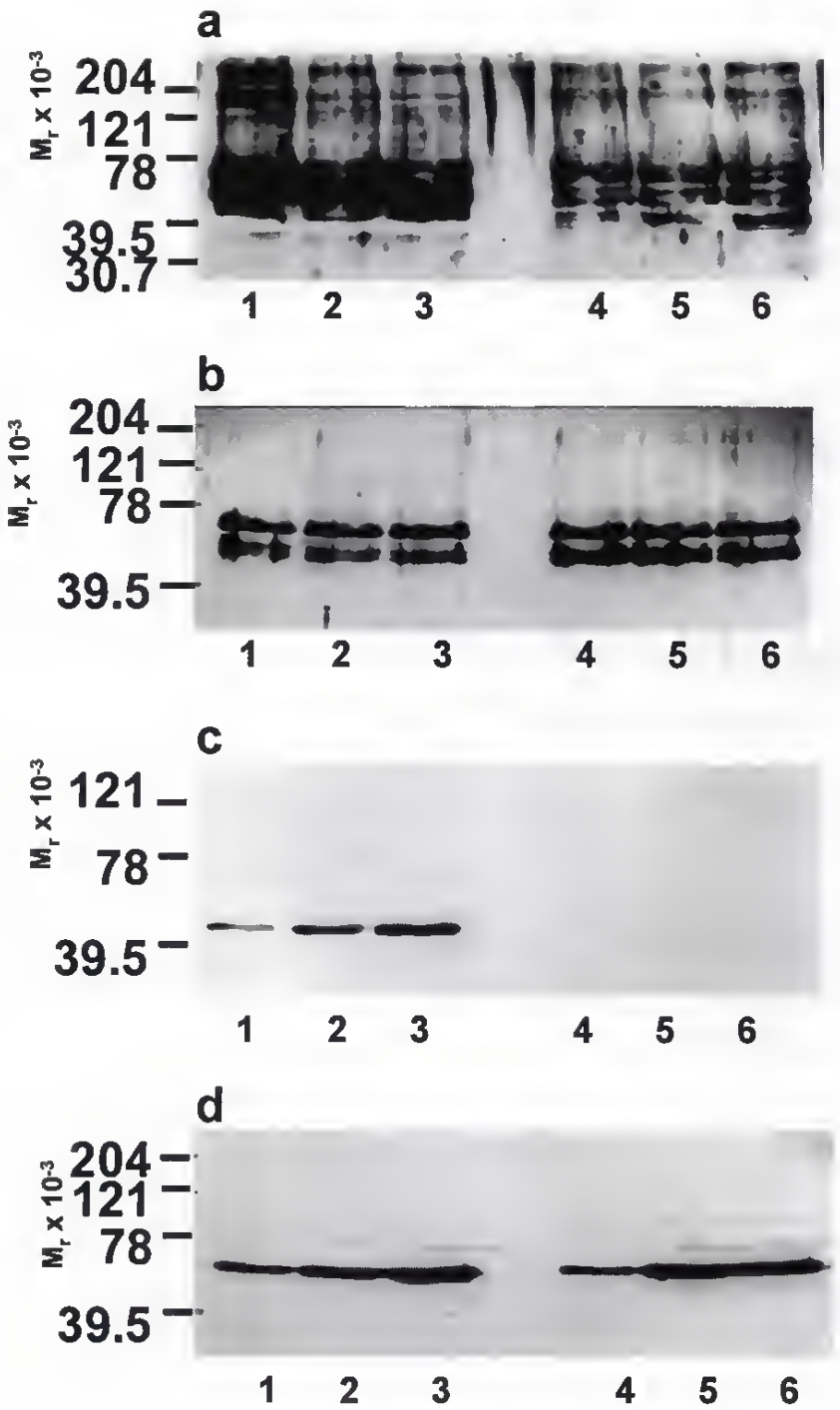


Figure 5-4. Immunoblotting analysis of **cytokeratin** and **vimentin** in whole cell extracts from BEND cells of different passages (P). Increasing concentrations of proteins from extracts (2.5, 5, 10 μg, lanes 1 and 4, 2 and 5, 3 and 6 respectively) were loaded.

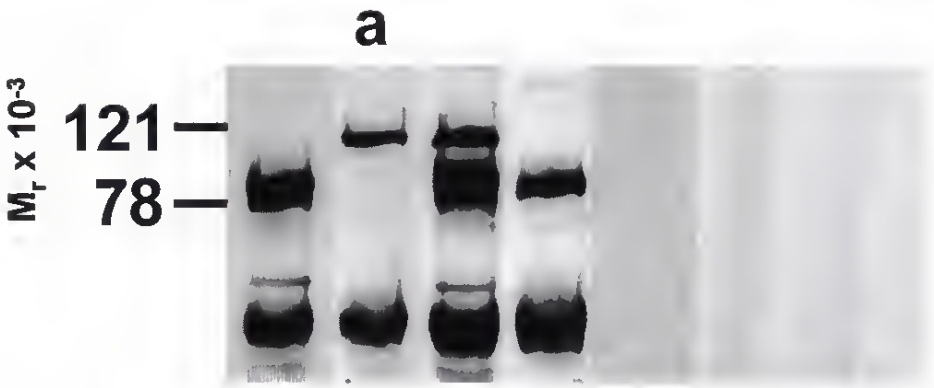
a) Cytokeratin, P9 (lanes 1-3), P2 (lanes 4-6); b) cytokeratin, P4 (lanes 1-3), P13 (lanes 4-6); c) Vimentin, P9 (lanes 1-3), P2 (lanes 4-6); d) vimentin, P4 (lanes 1-3), P13 (lanes 4-6).

Validation of Immunoprecipitation and Immunoblots

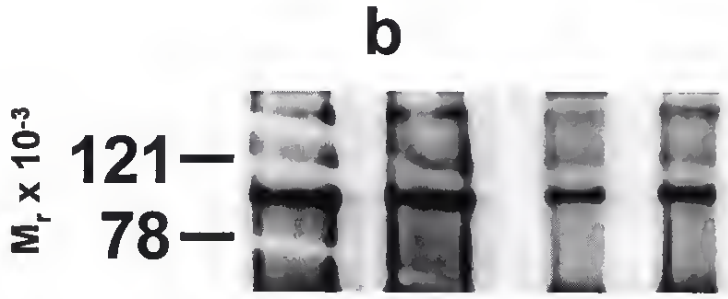
Immunoblots of STAT proteins immunoprecipitated with anti-STAT antibodies show a clear enrichment of the appropriate STAT, suggesting specificity (Figure 5-5). Moreover, no bands were detected when no antibody was used in the immunoprecipitation reaction, and only non-specific bands were present when samples were immunoprecipitated with normal rabbit serum (i.e., same banding pattern noted regardless of antibody used). Similarly, STAT bands were present in immunoblots probed with anti-phosphotyrosine and anti-STAT antibodies, but were not present when immunoblots were probed with no antibody or with normal rabbit serum (Figure 5-6).

Dose Response to bIFN–τ

Phosphorylation of STATs 1 and 2 increased with as little as 3.125 ng/ml of bIFN–τ for STAT-2 and 6.25 ng/ml for STAT-1 (Figure 5-7). There was a gradual increase in phosphorylation of STAT-1 that reached a maximum at a dose of 50 ng/ml and then declined. Phosphorylation of STAT-2 did not change significantly with doses of bIFN–τ greater than 3.125 ng/ml. Abundance of STATs 1 and 2 was unchanged across all doses. Based on these results, a dose of 50 ng/ml was chosen to be used in all subsequent experiments.



IP ab[a]	1	2	1/2	3	n	n	n	n
1[st] ab[b]	1	2	1/2	3	1	2	1/2	3
2[nd] ab[c]	r	r	r	r	r	r	r	r



IP ab[a]	NRS	NRS	NRS	NRS
1[st] ab[b]	1	1/2	2	3
2[nd] ab[c]	r	r	r	r

Figure 5-5. Validation of immunoprecipitation procedure on whole cell extracts (WCE) of BEND cells.

a) Enhanced chemiluminescence (ECL) exposure of WCE immunoprecipitated with anti-STAT-1, anti-STAT-2 , anti-STAT-3 or nothing; b) ECL exposure of WCE immunoprecipitated with normal rabbit serum.

[a] Antibody used for immunoprecipitation (1=anti-STAT-1; 2=anti-STAT-2; 3=anti-STAT-3; NRS=normal rabbit serum; n=nothing).

[b] First antibody used for immunoblotting (1=anti-STAT-1; 2=anti-STAT-2; 3=anti-STAT-3.

[c] Second antibody used for immunoblotting (r=anti-rabbit IgG).

Figure 5-6. Validation of immunoblotting procedure on whole cell extracts of BEND cells incubated for 15 minutes in presence (+) or absence (-) of bIFN-τ.

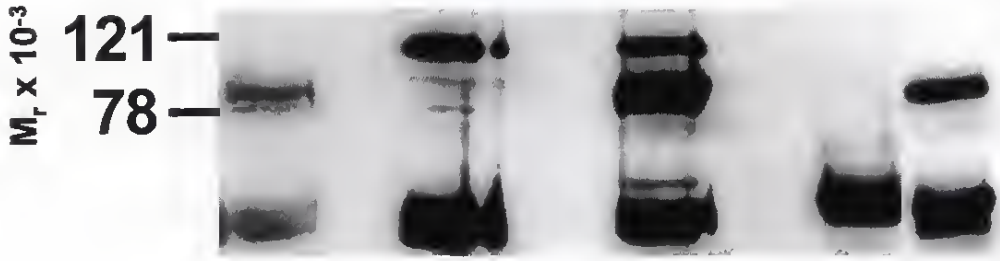
a) Enhanced chemiluminescence (ECL) exposure of abundance of STATs 1, 2 and 3; b) ECL exposure of tyrosine phosphorylation of STATs 1, 2 and 3.

[a] Antibody used for immunoprecipitation (1=anti-STAT-1; 2=anti-STAT-2; 3=anti-STAT-3).

[b] First antibody used for immunoblotting (1=anti-STAT-1; 2=anti-STAT-2; 3=anti-STAT-3; p-Y=anti-phosphotyrosine; NRS=normal rabbit serum; n=nothing).

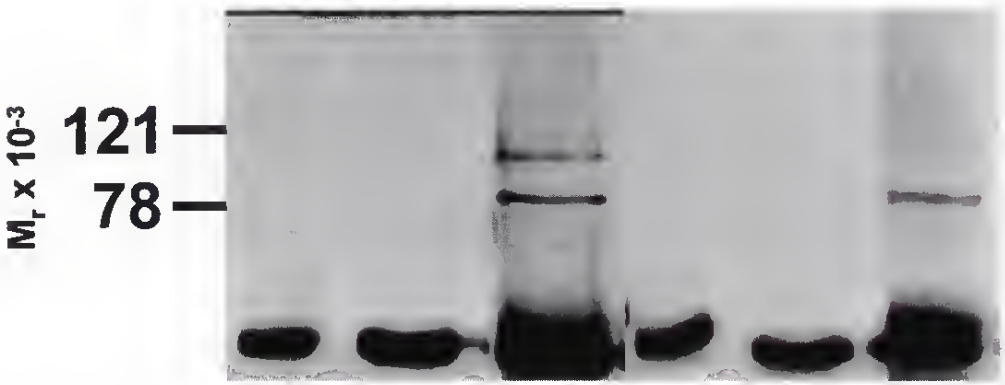
[c] Second antibody used for immunoblotting (r=anti-rabbit IgG; m=anti-mouse IgG; n=nothing).

a



bIFN-τ	-	-	-	-	-	-	-	-	-
IP ab[a]	1/2	1/2	1/2	1/2	1/2	1/2	1/2	3	3
1st ab[b]	1	1	2	2	1/2	1/2	NRS	3	3
2nd ab[c]	r	n	r	n	r	n	r	r	n

b



bIFN-τ	+	+	+	+	+	+
IP ab[a]	1/2	1/2	1/2	3	3	3
1st ab[b]	n	NRS	p-Y	n	NRS	p-Y
2nd ab[c]	m	m	m	m	m	m

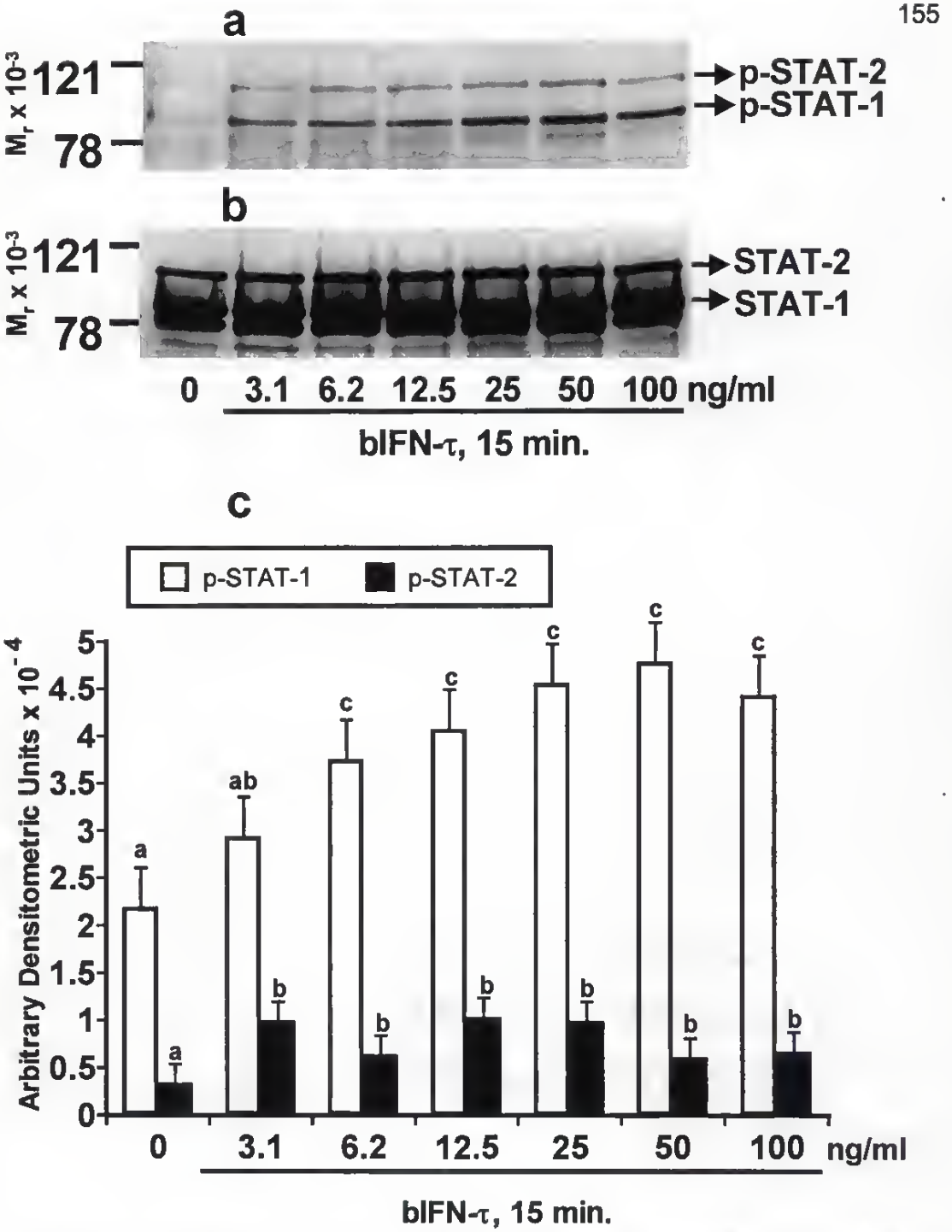


Figure 5-7. Immunoblotting analysis of **STATs 1 and 2 immunoprecipitated from whole cell extracts** from BEND cells treated with increasing doses of bIFN-τ for 15 minutes.

a) Representative enhanced chemiluminescence (ECL) exposure of tyrosine phosphorylation of STATs 1 and 2; b) Representative ECL exposure of abundance of STATs 1 and 2; c) Least squares means and SE of tyrosine phosphorylation of STATs 1 and 2 (within each variable, bars with distinct subscripts are statistically different, $p<0.1$).

Time Response to bIFN–τ

Bovine IFN–τ induced tyrosine phosphorylation of both STATs 1 and 2 (Figure 5-8) and STAT-3 (Figure 5-9) after as early as 3 minutes exposure. Maximum phosphorylation occurred at 8 minutes for STATs 1 and 2 but only at 15 minutes for STAT-3. For STATs 1, 2 and 3, phosphorylation gradually decreased to reach control levels by 60 minutes. Exposure to bIFN–τ did not affect abundance of STATs 1, 2 and 3.

Nuclear Translocation of STATs

STAT-1. Examination of Coomassie blue stained gels revealed differences in banding patterns between CE and NE, suggesting that different cellular fractions were obtained. Abundance of STAT-1 in CE remained constant regardless of the length of time of exposure to bIFN–τ (Figure 5-10). In contrast abundance of STAT-1 in NE increased by 8 minutes in response to bIFN-τ, reaching a maximum at 15 minutes, remained elevated and slightly decreased by 120 minutes (Figure 5-11). Bovine IFN–τ stimulated a transient increase in phosphorylation of STAT-1 in the cytosol that was first observed after 3 minutes, maintained maximum levels between 8 and 15 minutes, and decreased to control levels by 120 minutes (Figure 5-12). However, phosphorylation was delayed in the nucleus, and maximum phosphorylation only occurred after a 30

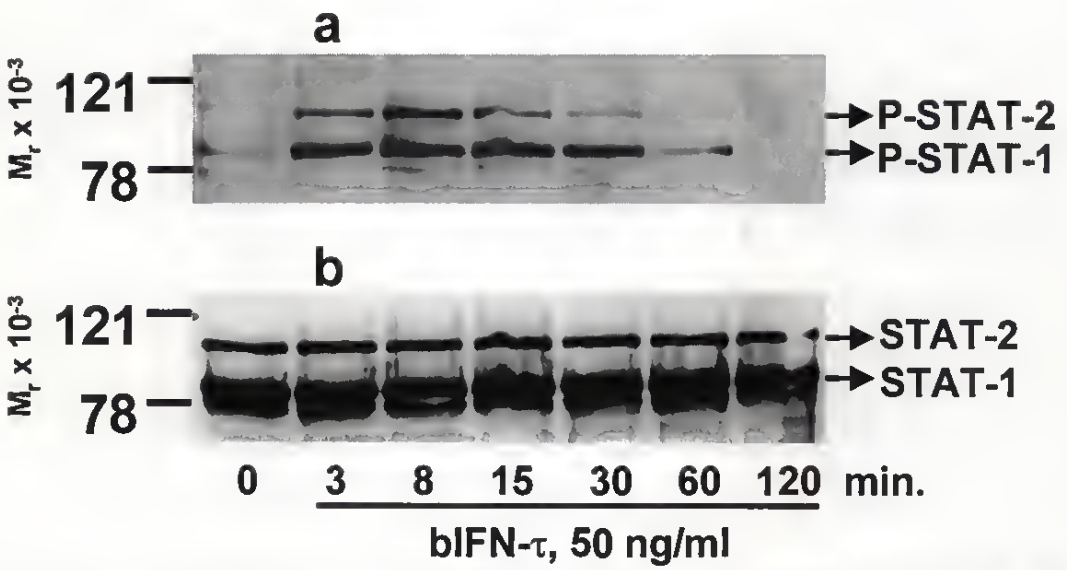


Figure 5-8. Immunoblotting analysis of **STATs 1 and 2 immunoprecipitated from whole cell extracts** from BEND cells treated with bIFN-τ for increasing intervals of time.
a) Representative enhanced chemiluminescence (ECL) exposure of tyrosine phosphorylation of STATs 1 and 2; b) Representative ECL exposure of abundance of STATs 1 and 2.

minute exposure to bIFN–τ (Figure 5-13). Phosphorylation decreased by 60 minutes.

STAT-2. In the cytosol there was a numerical (statistically non-significant) decrease in the abundance of STAT-2 that reached a minimum level at 30 minutes but returned to control levels by 120 minutes (Figure 5-10). Abundance of STAT-2 in NE started to increase after only 1 minute exposure to bIFN–τ and reached a maximum at 15 minutes, remaining elevated up to 30 minutes and then slowly decreased (Figure 5-11). Phosphorylation of STAT-2 in the cytosol increased after 3 minutes, remained elevated between 3 and 15 minutes and gradually decreased to levels lower than control levels in all other time points (Figure 5-12). Due to lack of sensitivity, phosphorylation of STAT-2 in the

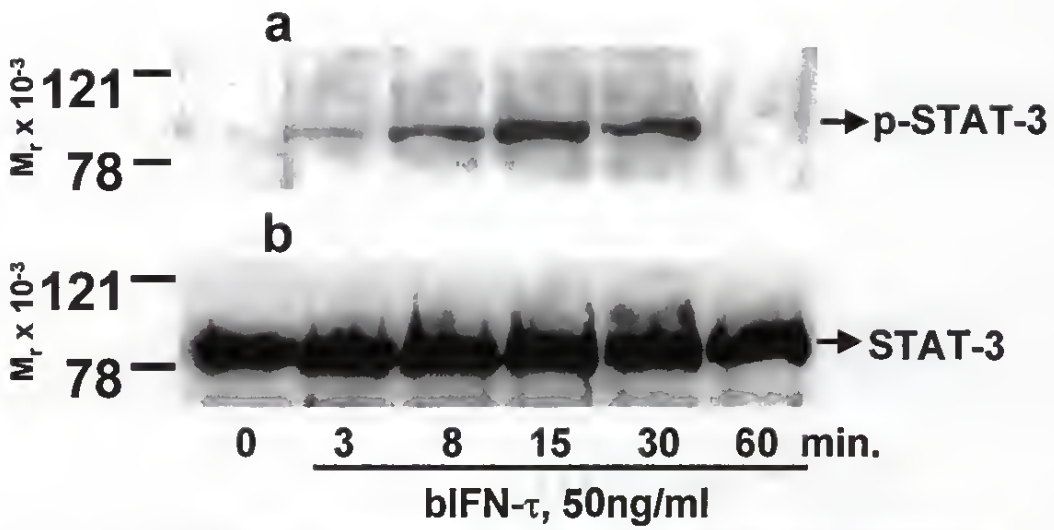


Figure 5-9. Immunoblotting analysis of **STAT-3 immunoprecipitated from whole cell extracts** from BEND cells treated with bIFN-τ for increasing intervals of time.
a) Representative enhanced chemiluminescence (ECL) exposure of tyrosine phosphorylation of STAT-3; b) Representative ECL exposure of abundance of STAT-3.

nucleus was only detected in one experiment, where it increased by 15 minutes and remained elevated for up to 120 minutes (Figure 5-13).

STAT-3. Similar to STATs 1 and 2, there was an increase in abundance of STAT-3 that lasted from 3 to 120 minutes in CE (Figure 5-14). In the nucleus, bIFN–τ increased abundance of STAT-3, which lasted from 8 to 60 minutes (Figure 5-15). Tyrosine phosphorylation of STAT-3 increased gradually to reach maximum levels at 15 minutes in the cytosol (Figure 5-16) and at 30 minutes in the nucleus (Figure 5-17). Levels of phosphorylation decreased abruptly after 60 and 120 minutes exposure to bIFN–τ.

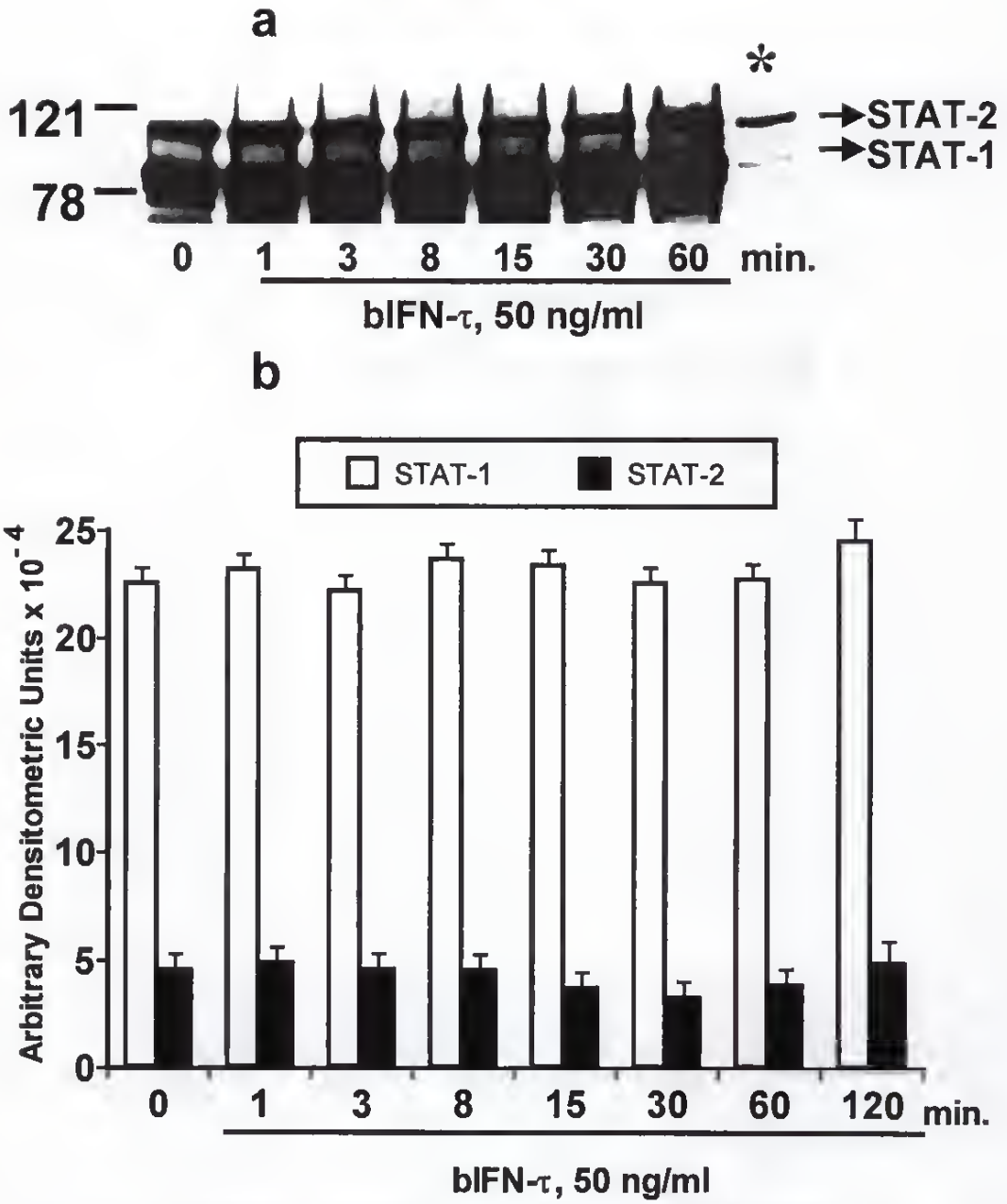


Figure 5-10. Immunoblotting analysis of **STATs 1 and 2 immunoprecipitated from cytosolic extracts** from BEND cells treated with bIFN-τ for increasing intervals of time.
a) Representative enhanced chemiluminescence (ECL) exposure of abundance of STATs 1 and 2; * represents positive control for STATs 1 and 2; b) Least squares means and SE of abundance of STATs 1 and 2.

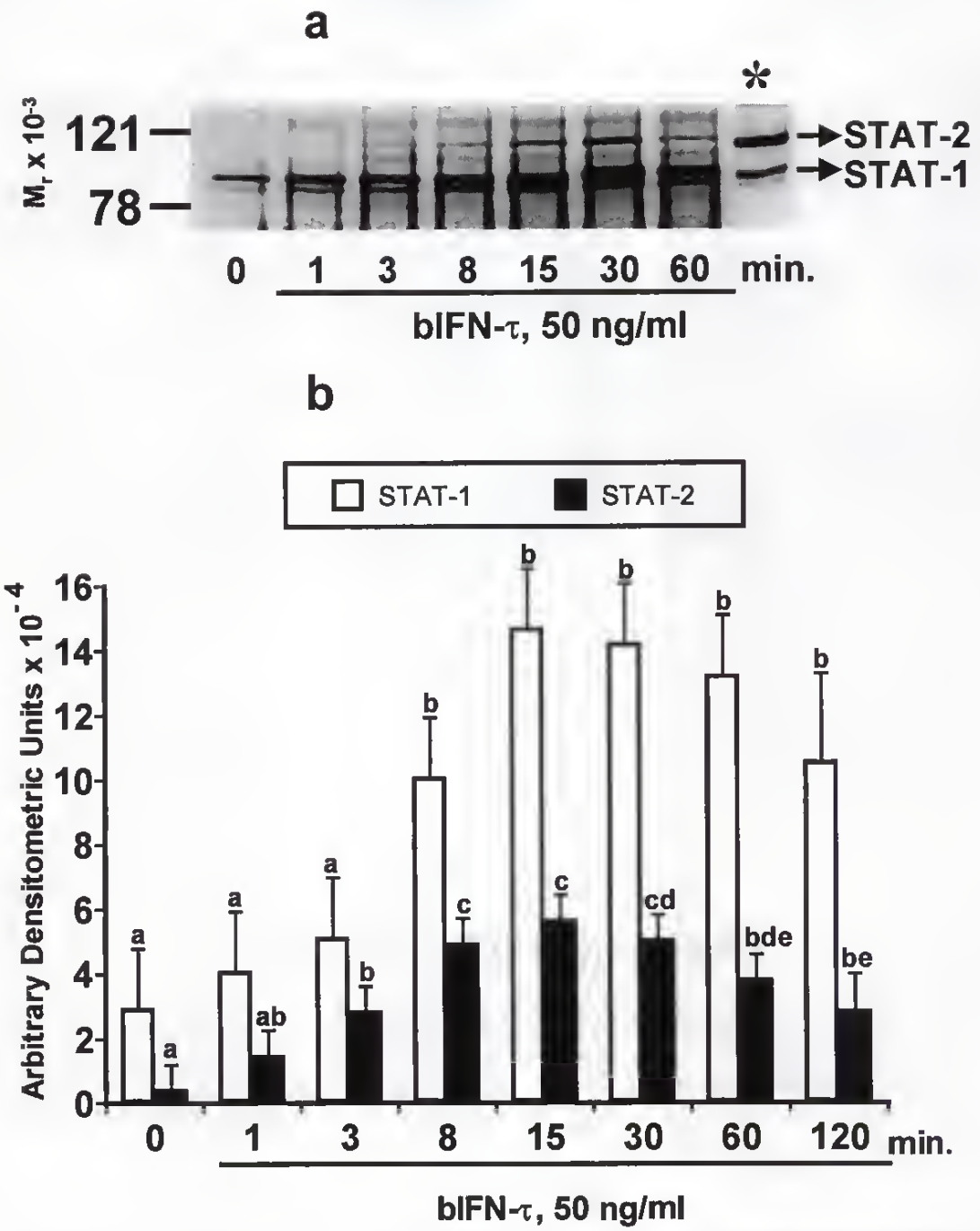


Figure 5-11. Immunoblotting analysis of **STATs 1 and 2 immunoprecipitated from nuclear extracts** from BEND cells treated with bIFN-τ for increasing intervals of time.

a) Representative enhanced chemiluminescence (ECL) exposure of abundance of STATs 1 and 2 (*, positive control) ; b) Least squares means and SE of abundance of STATs 1 and 2 (within each variable, bars with distinct subscripts are statistically different, $p<0.1$).

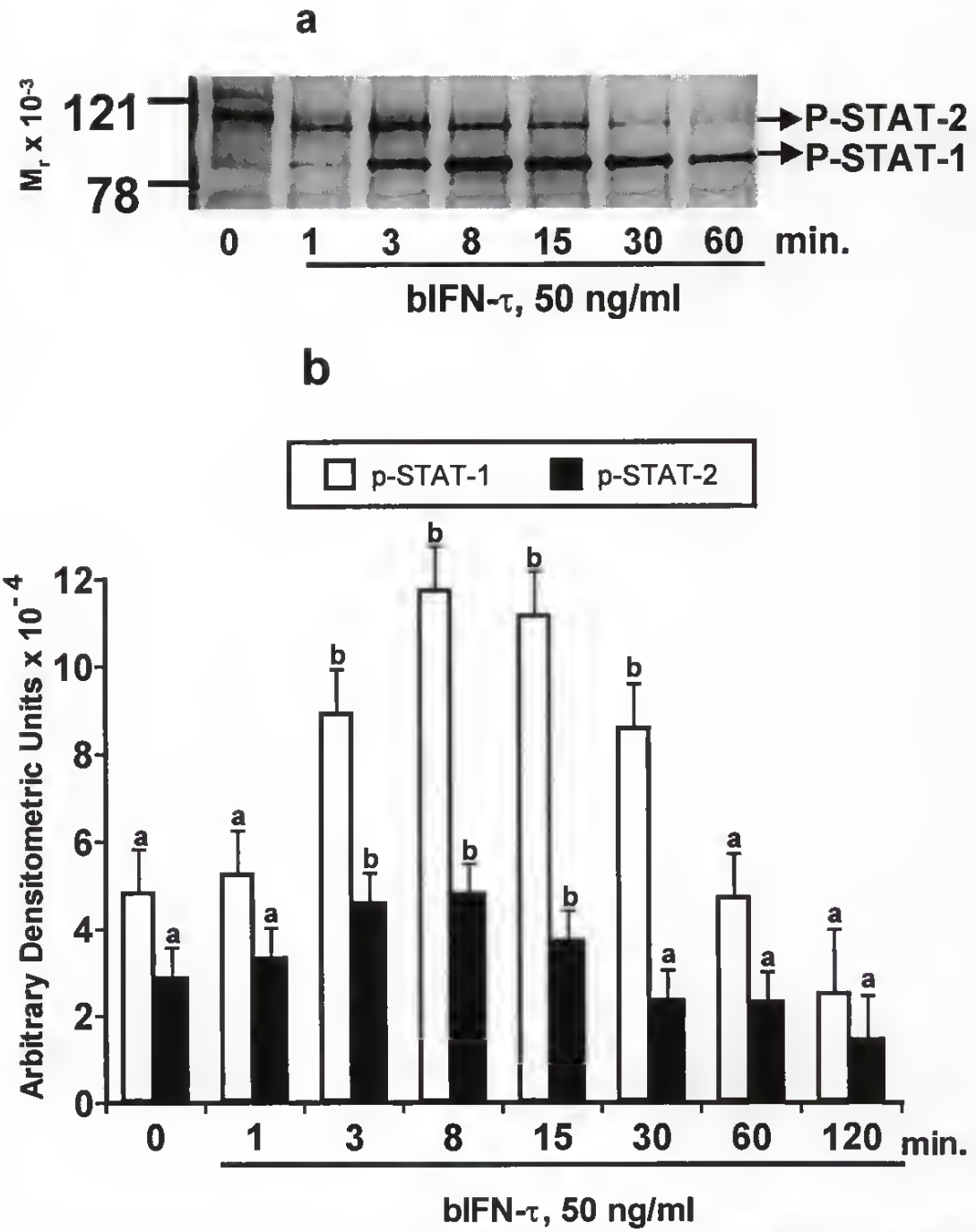


Figure 5-12. Immunoblotting analysis of **STATs 1 and 2 immunoprecipitated from cytosolic extracts** from BEND cells treated with bIFN-τ for increasing intervals of time.

a) Representative enhanced chemiluminescence (ECL) exposure of tyrosine phosphorylation of STATs 1 and 2; b) Least squares means and SE of tyrosine phosphorylation of STATs 1 and 2 (within each variable, bars with distinct subscripts are statistically different, $p<.1$).

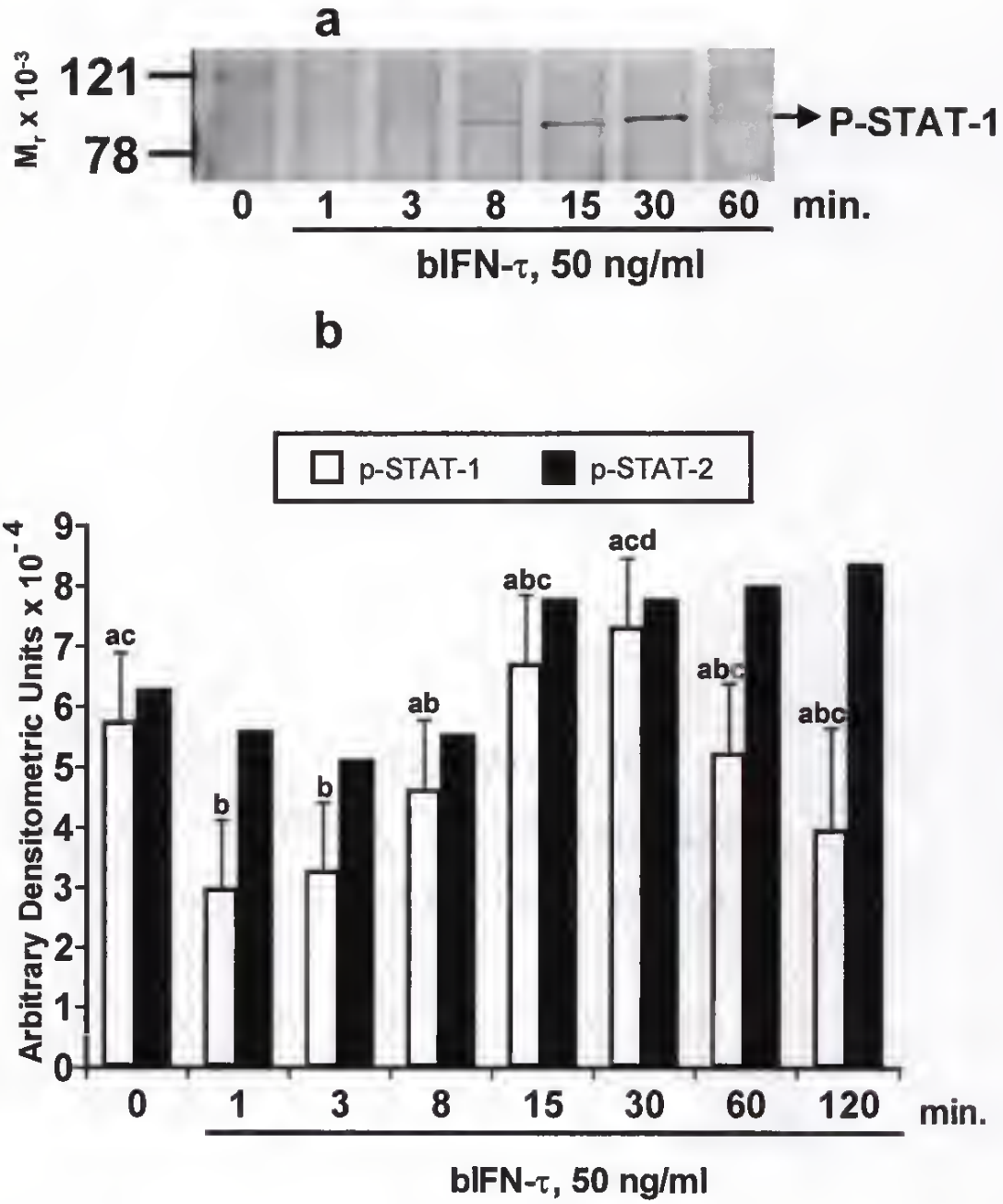


Figure 5-13. Immunoblotting analysis of **STATs 1 and 2 immunoprecipitated from nuclear extracts** from BEND cells treated with bIFN-τ for increasing intervals of time.

a) Representative enhanced chemiluminescence (ECL) exposure of tyrosine phosphorylation of STATs 1 and 2; b) Least squares means and SE of tyrosine phosphorylation of STATs 1 and 2 (within each variable, bars with distinct subscripts are statistically different, $p<.1$).

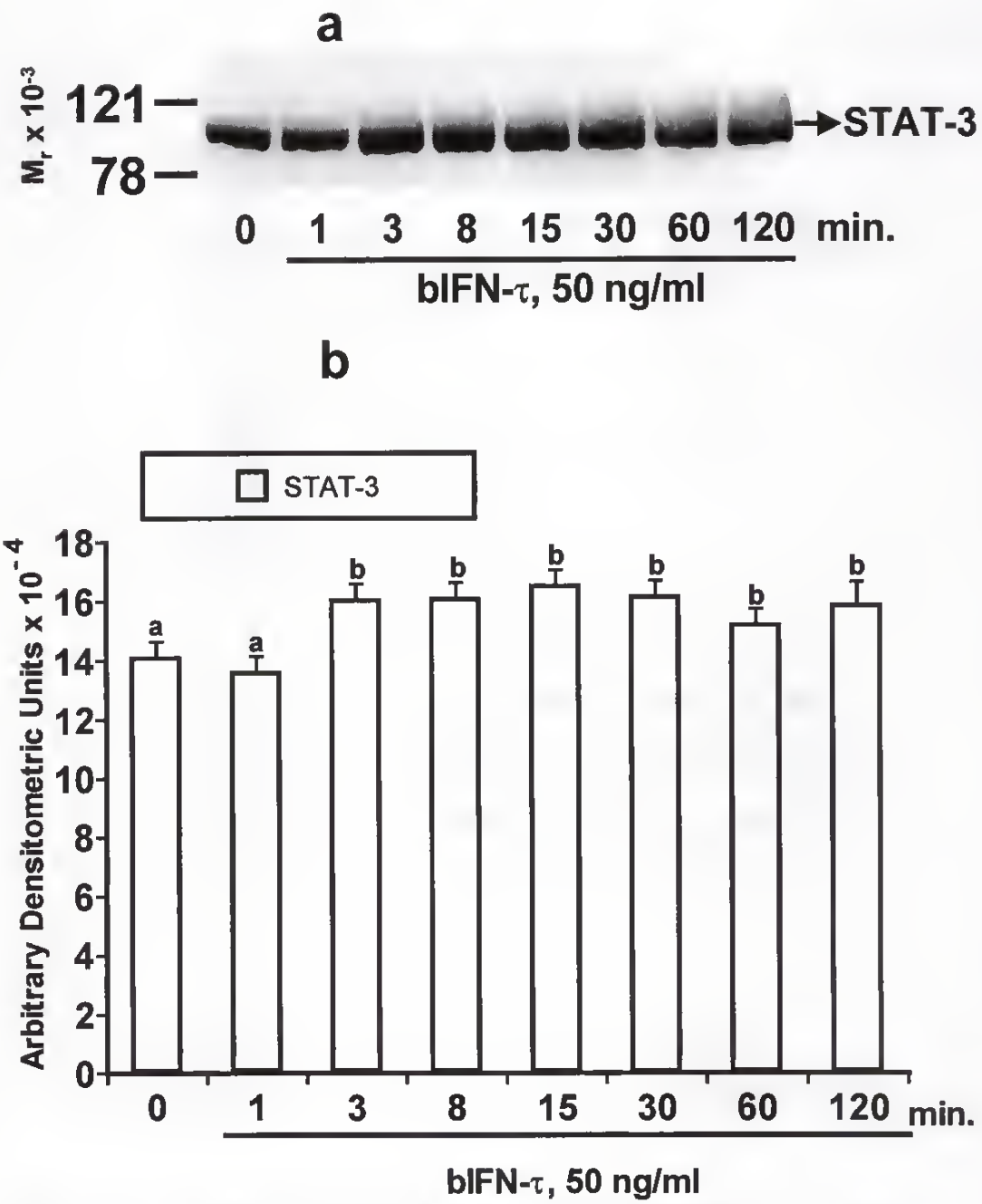


Figure 5-14. Immunoblotting analysis of **STATs 3 immunoprecipitated from cytosolic extracts** from BEND cells treated with bIFN-τ for increasing intervals of time.

a) Representative enhanced chemiluminescence (ECL) exposure of abundance of STAT-3; b) Least squares means and SE of abundance of STAT-3 (bars with distinct subscripts are statistically different, $p<0.1$).

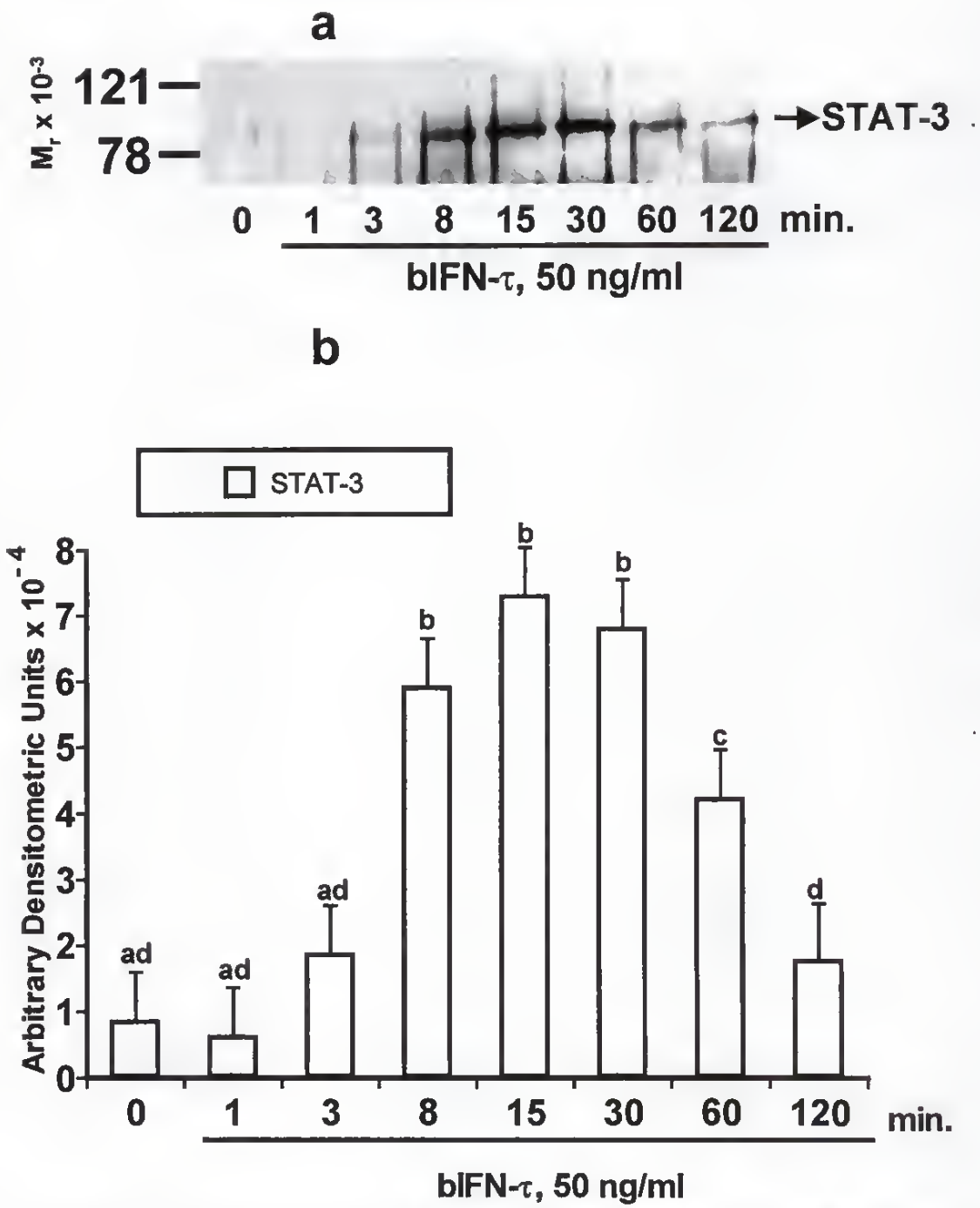


Figure 5-15. Immunoblotting analysis of **STATs 3 immunoprecipitated from nuclear extracts** from BEND cells treated with bIFN-τ for increasing intervals of time.

a) Representative enhanced chemiluminescence (ECL) exposure of abundance of STAT-3; b) Least squares means and SE of abundance of STAT-3 (bars with distinct subscripts are statistically different, $p<0.1$).

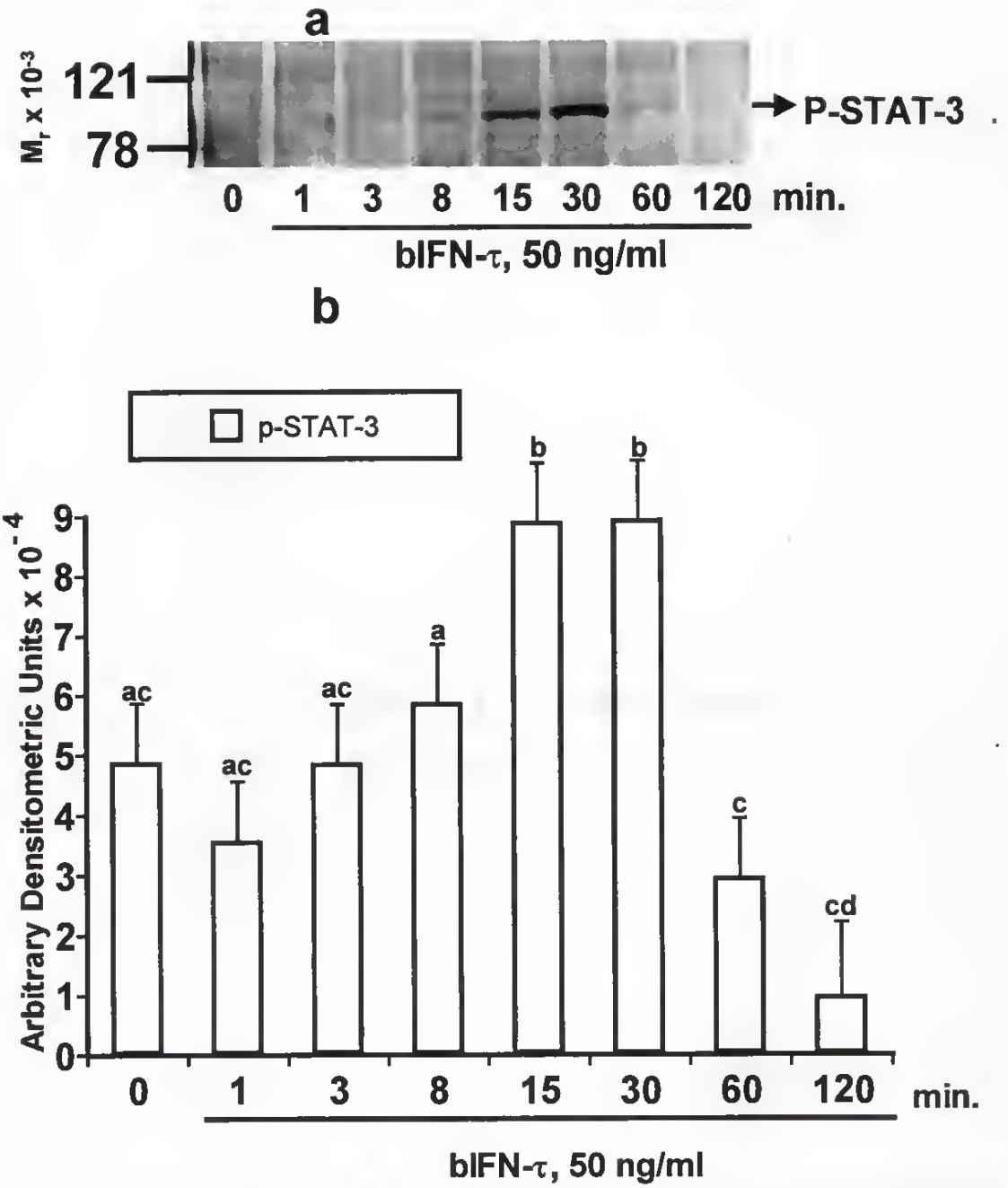

a
M_r x 10^{-3}
121
78
P-STAT-3
0 1 3 8 15 30 60 120 min.
bIFN-τ, 50 ng/ml
b
p-STAT-3
Arbitrary Densitometric Units x 10^{-4}
0 1 2 3 4 5 6 7 8 9
ac ac ac a b b c cd
0 1 3 8 15 30 60 120 min.
bIFN-τ, 50 ng/ml

Figure 5-16. Immunoblotting analysis of **STATs 3 immunoprecipitated from cytosolic extracts** from BEND cells treated with bIFN-τ for increasing intervals of time.

a) Representative enhanced chemiluminescence (ECL) exposure of tyrosine phosphorylation of STAT-3 ; b) Least squares means and SE of tyrosine phosphorylation of STAT-3 (bars with distinct subscripts are statistically different, $p<.1$).

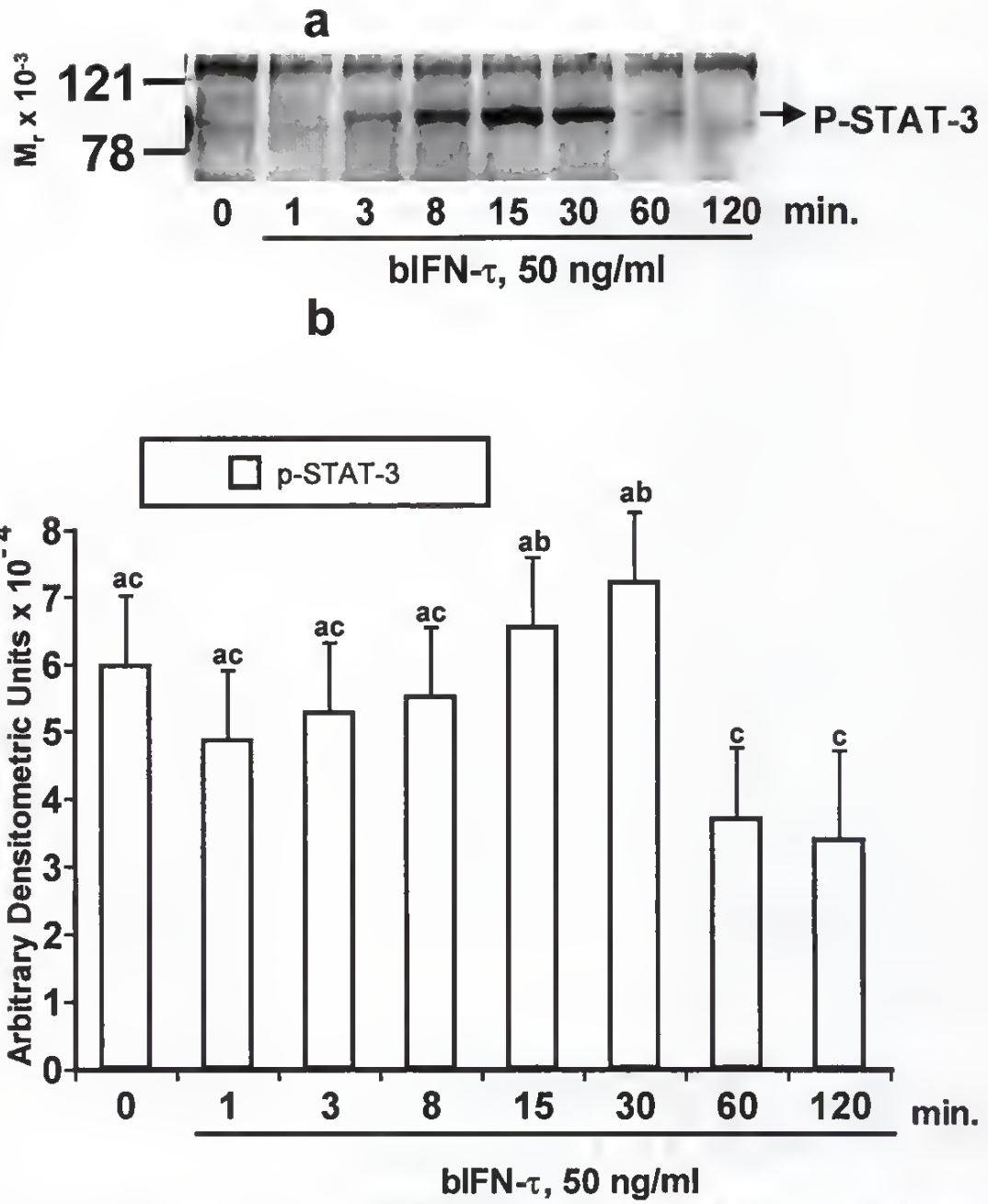


Figure 5-17. Immunoblotting analysis of **STATs 3 immunoprecipitated from nuclear extracts** from BEND cells treated with bIFN-τ for increasing intervals of time.

a) Representative enhanced chemiluminescence (ECL) exposure of tyrosine phosphorylation of STAT-3 ; b) Least squares means and SE of tyrosine phosphorylation of STAT-3 (bars with distinct subscripts are statistically different, $p<0.1$).

Validation of Time Responses to bIFN–τ

In the absence of bIFN–τ, there was no change in phosphorylation of STATs 1, 2 or 3 over time (Figure 5-18). Basal levels of phosphorylation associated with STAT proteins were noted, but there was no change in phosphorylation associated with time of exposure to medium alone.

Coimmunoprecipitation of STATs

Coimmunoprecipitation of STAT-1 with STAT-3 (immunoblot analysis of STAT-1 after immunoprecipitation of extracts with STAT-3 antibody). In CE, abundance of STAT-1 complexed with STAT-3 decreased during the 30 and 60 minutes samples, but increased again by 120 minutes (Figure 5-19). There was an increase in abundance of STAT-1 associated with STAT-3 in the nucleus after 8 minutes of exposure to bIFN–τ (Figure 5-20). STAT-1 remained elevated up to 30 minutes and then declined after that.

Coimmunoprecipitation of STAT-2 with STAT-3. In the cytosol, abundance of STAT-2 gradually decreased to reach its minimum level at 30 minutes, but increased to control levels at 120 minutes (Figure 5-19). In NE, bIFN–τ stimulated a time-dependent association of STAT-2 with STAT-3 that increased after 8 minutes to reach a maximum at 15 minutes and decline thereafter (Figure 5-20).

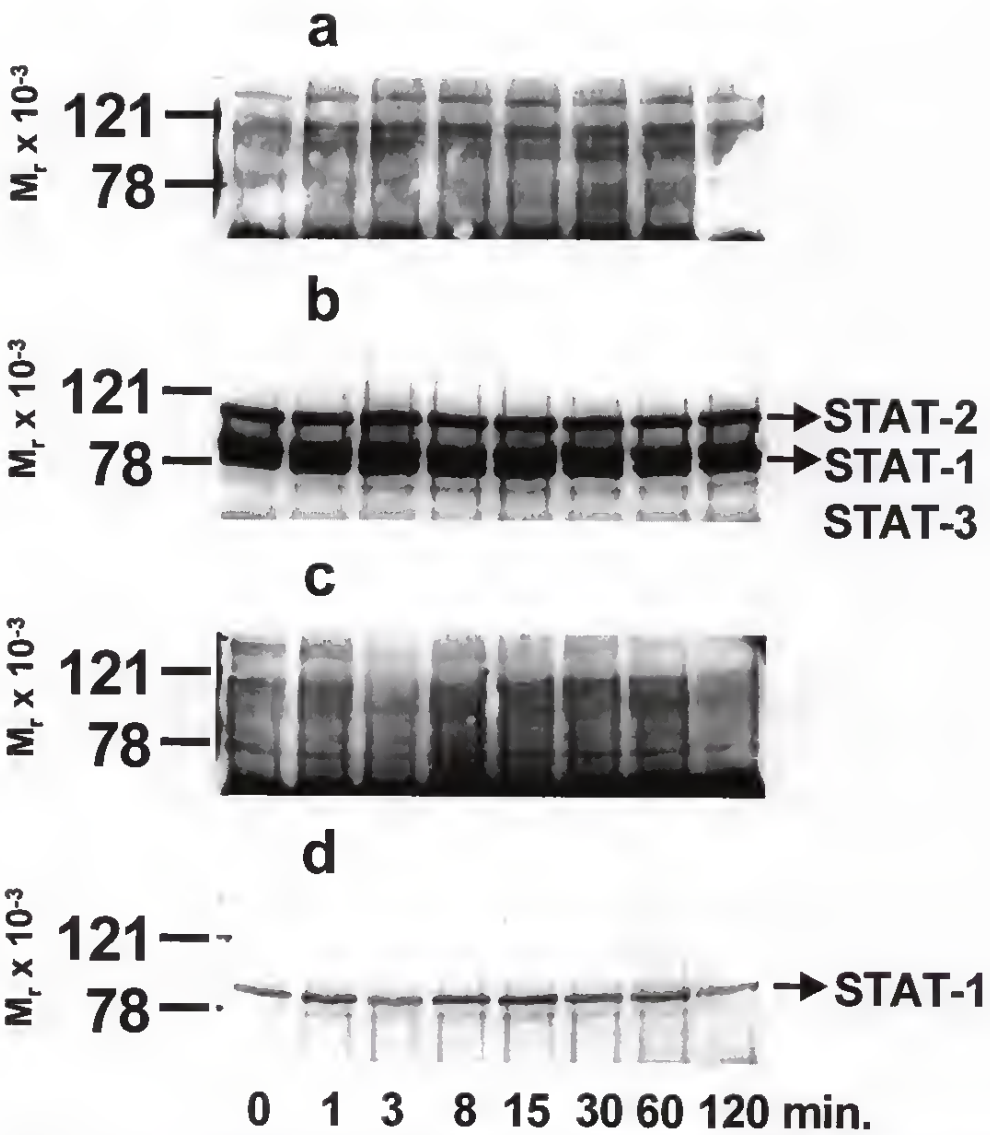


Figure 5-18. Verification of effects of time on **phosphorylation of STATs 1 and 2 in cytosolic (CE) and nuclear extracts (NE)** from BEND cells incubated with medium alone for increasing intervals of time.
a) Enhanced chemiluminescence (ECL) exposure of tyrosine phosphorylation of STATs 1, 2 and 3 in CE; b) ECL exposure of abundance of STATs 1, 2 and 3 in CE; c) ECL exposure of tyrosine phosphorylation of STATs 1, 2 and 3 in NE; d) ECL exposure of abundance of STATs 1, 2 and 3 in CE.

Figure 5-19. Immunoblotting analysis of **STATs 1 and 2 co-immunoprecipitated with STAT-3 from cytosolic extracts** from BEND cells treated with bIFN-τ for increasing intervals of time.

a) Representative enhanced chemiluminescence (ECL) exposure of abundance of STATs 1 and 2; b) Representative ECL exposure of tyrosine phosphorylation of STAT-3; c) Representative ECL exposure of abundance of STAT-3; d) Least squares means and SE of abundance of STATs 1 and 2 (within each variable, bars with distinct subscripts are statistically different, $p<.1$).

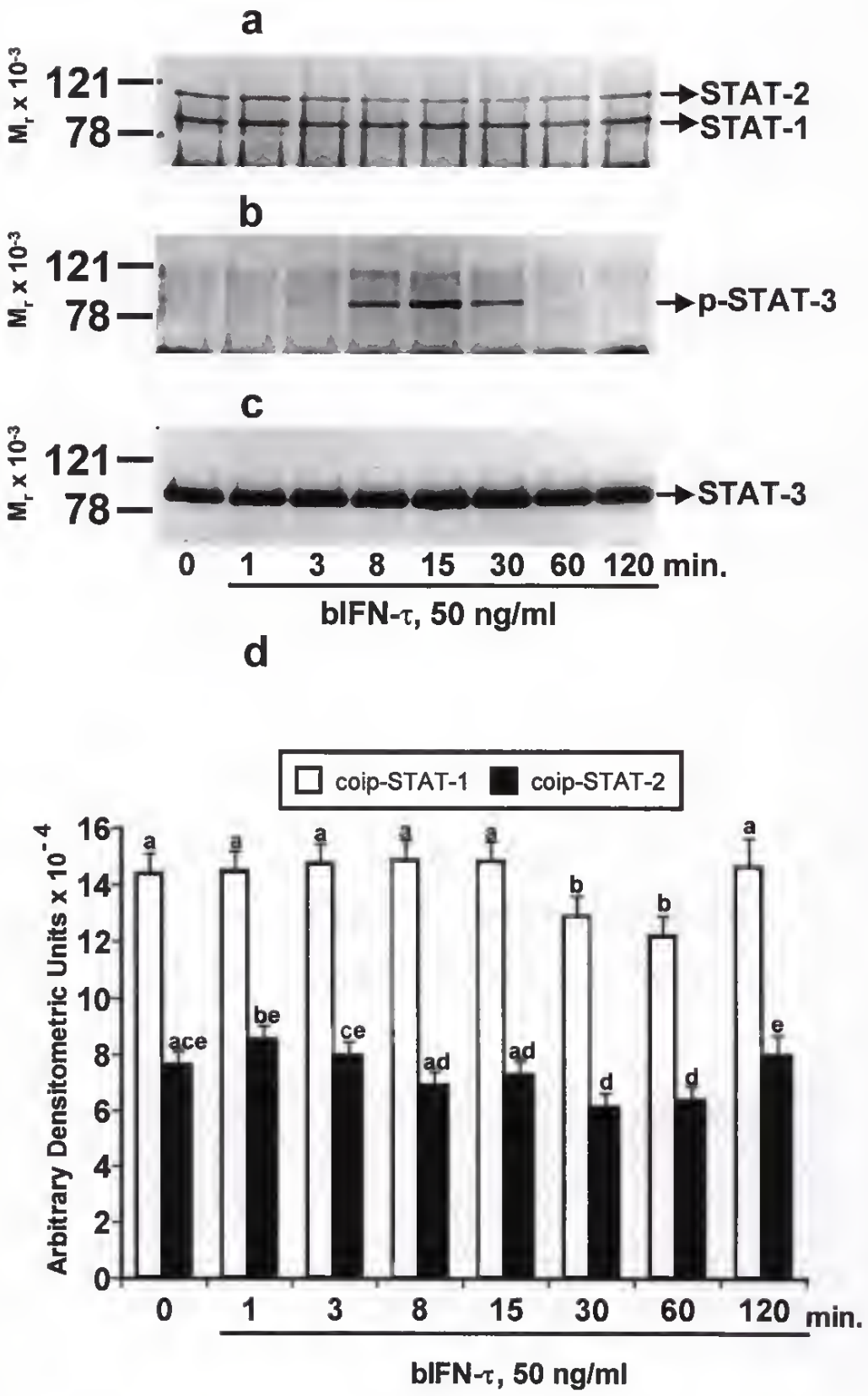
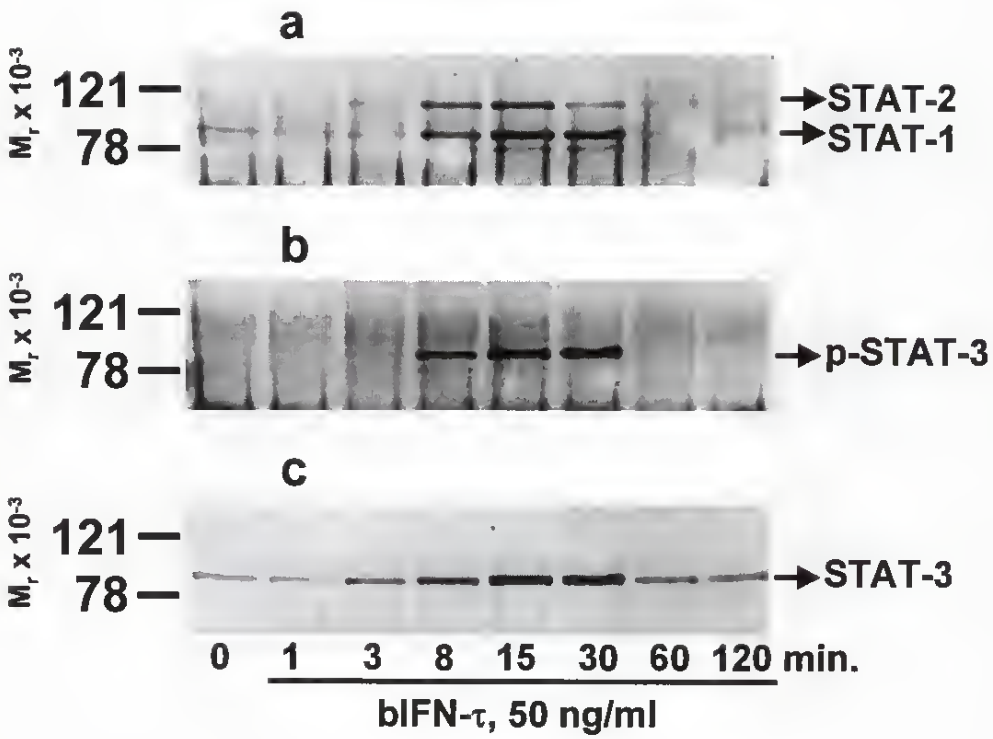
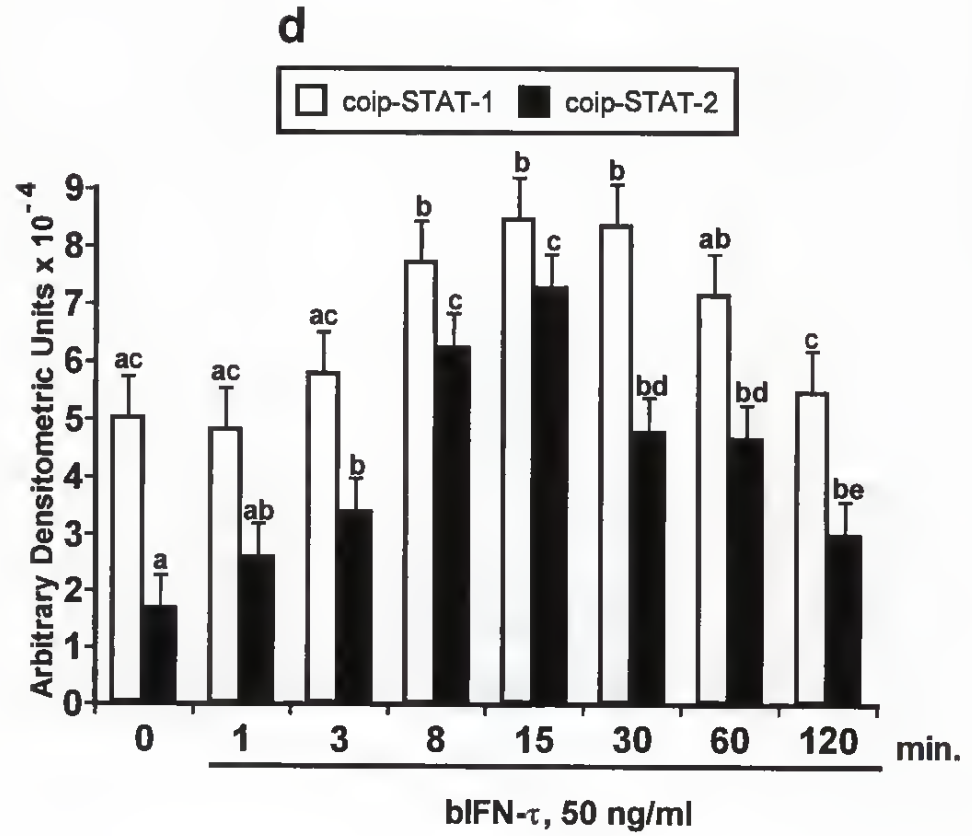

a
M_r x 10^{-3}
121
78
STAT-2
STAT-1
b
M_r x 10^{-3}
121
78
p-STAT-3
c
M_r x 10^{-3}
121
78
STAT-3
0 1 3 8 15 30 60 120 min.
bIFN-τ, 50 ng/ml
d
coip-STAT-1
coip-STAT-2
Arbitrary Densitometric Units x 10^{-4}
16
14
12
10
8
6
4
2
0
a
ace
a
be
a
ce
a
ad
a
ad
b
d
b
d
a
e
0 1 3 8 15 30 60 120 min.
bIFN-τ, 50 ng/ml

Figure 5-20. Immunoblotting analysis of **STATs 1 and 2 co-immunoprecipitated with STAT-3 from nuclear extracts** from BEND cells treated with bIFN-τ for increasing intervals of time.

a) Representative enhanced chemiluminescence (ECL) exposure of abundance of STATs 1 and 2; b) Representative ECL exposure of tyrosine phosphorylation of STAT-3; c) Representative ECL exposure of abundance of STAT-3; d) Least squares means and SE of abundance of STATs 1 and 2 (within each variable, bars with distinct subscripts are statistically different, $p<.1$).


a
M_r x 10^{-3}
121
78
STAT-2
STAT-1
b
M_r x 10^{-3}
121
78
p-STAT-3
c
M_r x 10^{-3}
121
78
STAT-3
0 1 3 8 15 30 60 120 min.
bIFN-τ, 50 ng/ml


d
coip-STAT-1
coip-STAT-2
Arbitrary Densitometric Units x 10^{-4}
0 1 2 3 4 5 6 7 8 9
ac a ac ab ac b b c b c b c ab bd ab bd c be
0 1 3 8 15 30 60 120 min.
bIFN-τ, 50 ng/ml

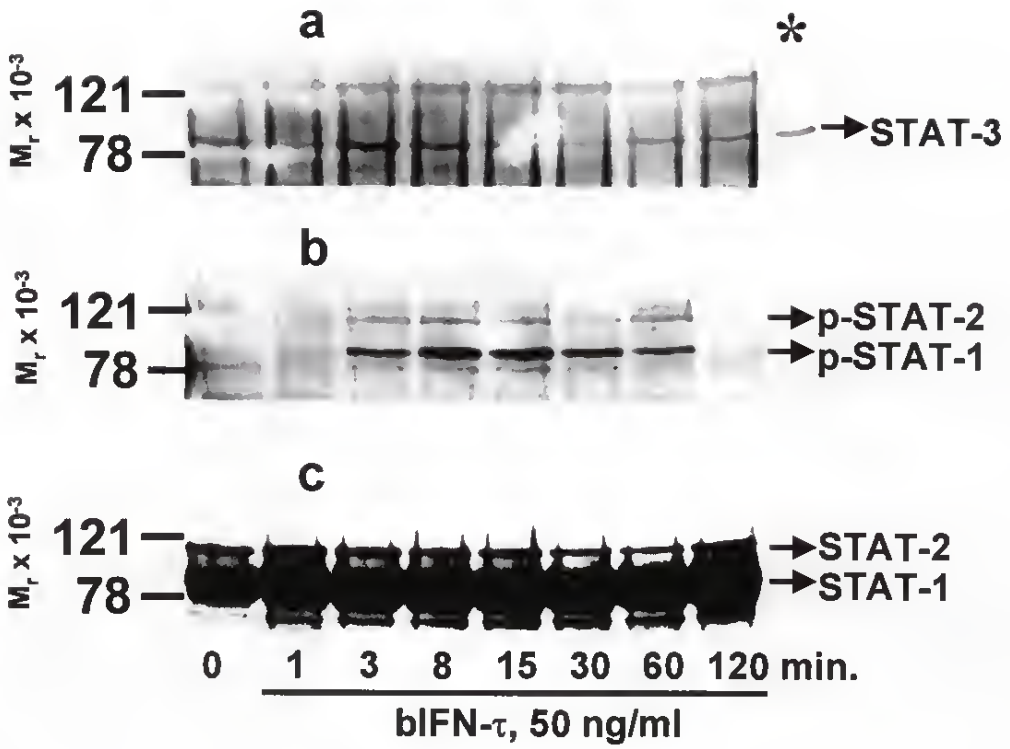
Coimmunoprecipitation of STAT-3 with STATs 1 and 2. There was a numerical decrease in the abundance of STAT-3 in the cytosol at 15 minutes, but such decrease may have been due to lower densitometric reading because of a "bubble" (see Figure 5-21). Abundance remained lower up to 120 minutes (Figure 5-21). In the nucleus, STAT-3 association with STATs 1 and / or 2 increased overtime to reach maximum levels at 15 minutes and then gradually decreased (Figure 5-22).

Discussion

Presence and activation of tyrosine phosphorylation of STAT proteins by bIFN–τ has been shown previously in non-reproductive tissues (MDBK cells; Subramaniam et al., 1995) as well as bovine uterus (Binelli et al., 1996; Perry et al., 1999; Chapter 4). The present series of experiments demonstrate that bIFN–τ can stimulate the JAK-STAT pathway of signal transduction in a line of bovine endometrial epithelial cells (BEND cells; Austin et al., 1996b). The STAT proteins 1, 2 and 3 are present, and after exposure to bIFN–τ become tyrosine-phosphorylated in a dose- and time-dependent manner and associate to form complexes. Such complexes migrate to the nucleus of cells, where they can act as transcription factors and induce or repress expression of IFN-responsive genes (Figure 5-23).

Figure 5-21. Immunoblotting analysis of **STAT-3 co-immunoprecipitated with STATs 1 and 2 from cytosolic extracts** from BEND cells treated with bIFN-τ for increasing intervals of time.

a) Representative enhanced chemiluminescence (ECL) exposure of abundance of STAT-3; b) Representative ECL exposure of tyrosine phosphorylation of STATs 1 and 2; c) Representative ECL exposure of abundance of STATs 1 and 2; d) Least squares means and SE of abundance of STAT-3 (bars with distinct subscripts are statistically different, $p<.1$).


a
*
M_r x 10^{-3}
121
78
STAT-3
b
M_r x 10^{-3}
121
78
p-STAT-2
p-STAT-1
c
M_r x 10^{-3}
121
78
STAT-2
STAT-1
0 1 3 8 15 30 60 120 min.
bIFN-τ, 50 ng/ml

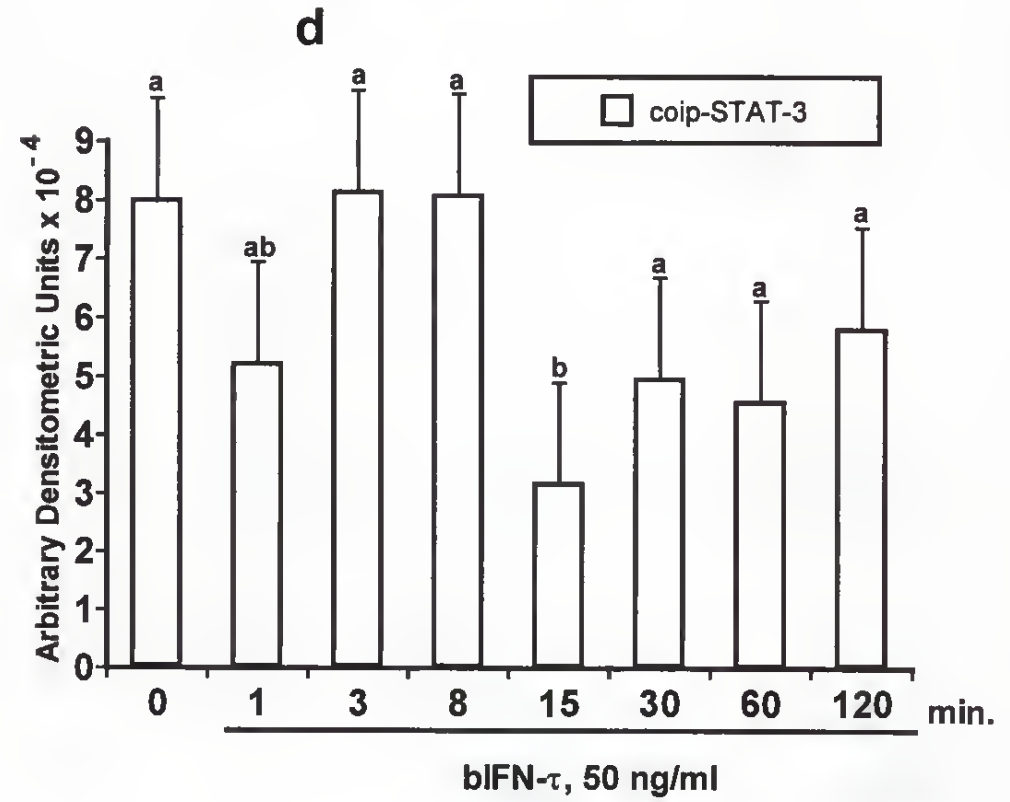
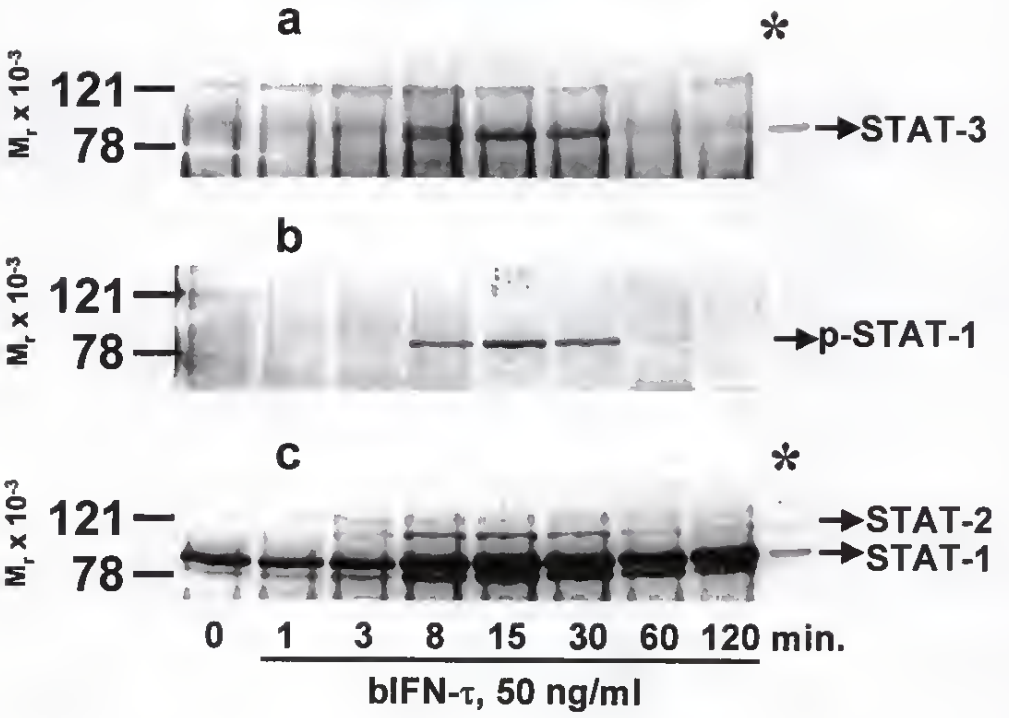
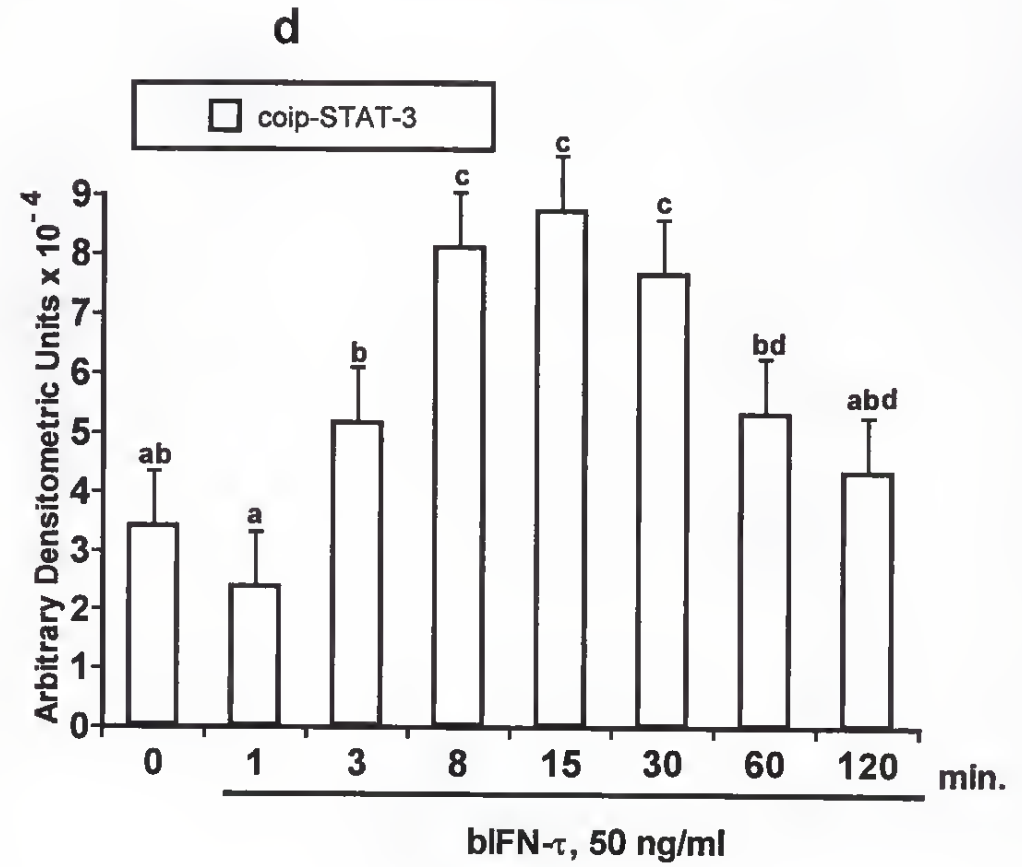

d
coip-STAT-3
Arbitrary Densitometric Units x 10^{-4}
0 1 2 3 4 5 6 7 8 9
a ab a a b a a a
0 1 3 8 15 30 60 120 min.
bIFN-τ, 50 ng/ml

Figure 5-22. Immunoblotting analysis of **STAT-3 co-immunoprecipitated with STATs 1 and 2 from nuclear extracts** from BEND cells treated with bIFN-τ for increasing intervals of time.

a) Representative enhanced chemiluminescence (ECL) exposure of abundance of STAT-3;. b) Representative ECL exposure of tyrosine phosphorylation of STATs 1 and 2; c) Representative ECL exposure of abundance of STATs 1 and 2; d) Least squares means and SE of abundance of STAT-3 (bars with distinct subscripts are statistically different, $p<0.1$).


a
*
M_r x 10^{-3}
121
78
STAT-3
b
M_r x 10^{-3}
121
78
p-STAT-1
c
*
M_r x 10^{-3}
121
78
STAT-2
STAT-1
0 1 3 8 15 30 60 120 min.
bIFN-τ, 50 ng/ml


d
coip-STAT-3
Arbitrary Densitometric Units x 10^{-4}
0 1 2 3 4 5 6 7 8 9
ab
a
b
c
c
c
bd
abd
0 1 3 8 15 30 60 120 min.
bIFN-τ, 50 ng/ml

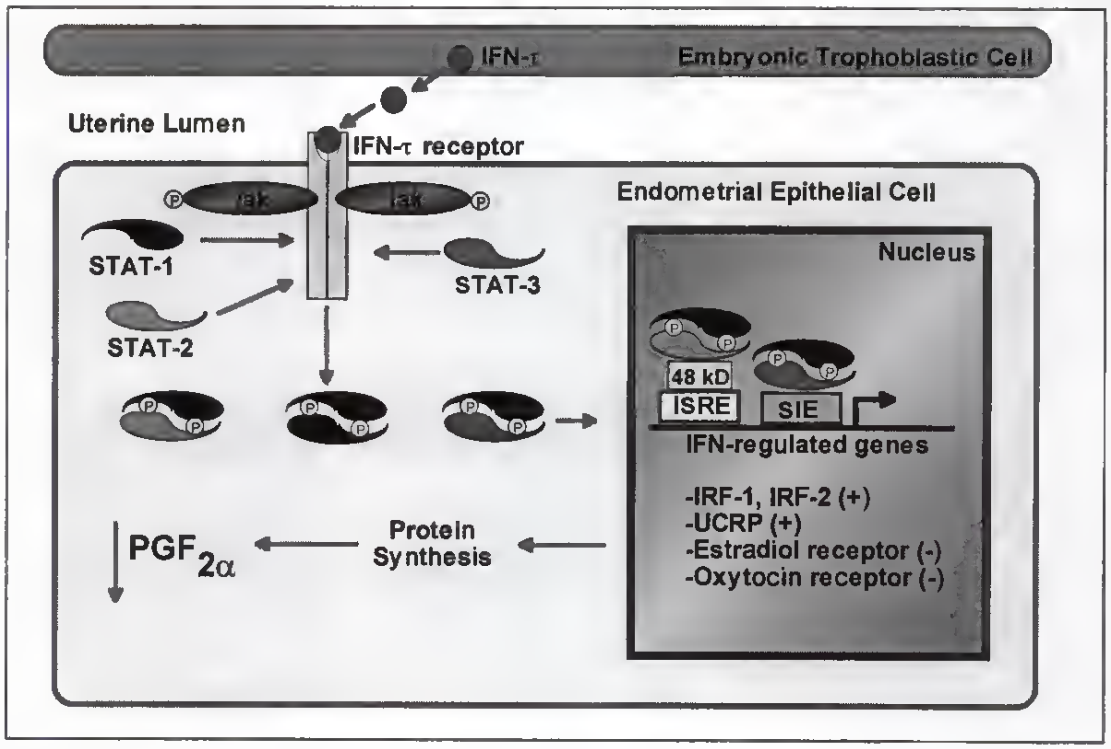


Figure 5-23. Hypothetical model for the bIFN-τ-stimulated JAK-STAT pathway of signal transduction in endometrial epithelial cells. Trophoblastic cells on the conceptus secrete bIFN-τ into the uterine lumen, and bIFN-τ interacts with its receptor in the apical aspect of endometrial epithelial cells. Binding of bIFN-τ elicits tyrosine phsphorylation, homo- and hetero-dimer formation of pre-existing, unphosphorylated STAT proteins. Dimers of STATs translocate to the nucleus where they bind to specific cis-activating elements [sis-inducible element (SIE) and interferon-stimulus response element (ISRE)] present in the regulatory region of interferon-regulated genes to stimulate their transcription. It is hypothesized that proteins induced by bIFN-τ, such as ubiquitin cross-reactive protein (UCRP), interferon regulatory factor (IRF)-1 and IRF-2 may act to suppress synthesis of $PGF_{2\alpha}$ from endometrial cells. Also, the JAK-STAT pathway may suppress synthesis of proteins involved the $PGF_{2\alpha}$ synthesis machinery, such as receptors for oxytocin and estradiol.

The BEND cells used in our studies are a spontaneously immortalized cell line, derived from endometrial epithelial cells of cows on day 14 of the estrous cycle (Austin et al., 1996a). Similarly to endometrial explants, BEND cells secrete both UCRP and GCP-2 in response to bIFN–τ (Austin et al., 1996b; Teixeira et al., 1997; Johnson et al., 1998a; Staggs et al., 1998), suggesting that they are an adequate model for studying IFN-stimulated signal transduction.

Due to the relative low abundance of STAT proteins in cells, the immunoprecipitation approach was chosen to concentrate STATs and facilitate detection and analysis of STATs in immunoblots. The anti-human STAT antibodies specifically immunoprecipitated and detected bands for bovine STATs 1, 2 and 3, based on their predicted molecular weight on immunoblots. Therefore, all experiments were conducted using anti-human STAT antibodies.

The initial objective of the present study was to determine a dose of bIFN-τ to be used in the experiments. To my best knowledge, no precise measurement of concentrations of bIFN-τ in the uterus have been taken. Trophoblastic cells on the surface of the elongating embryo secrete bIFN-τ locally, and bIFN-τ is probably readily taken up by epithelial cells in the endometrium. Therefore, measurements of concentrations of bIFN-τ in uterine fluid would probably be meaningless. One strategy to estimate physiological doses of bIFN-τ is to examine the Kd for the IFN-τ receptor. Based on the calculated Kd for the ovine and bIFN–τ receptor in endometrium, the physiological dose for bIFN–τ would be between .2 and 11.6 ng/ml (discussed in Danet-Desnoyers et al., 1994), which is lower than the dose that elicited maximum phosphorylation of STAT-1 (50 ng/ml). Compared to other experiments utilizing bIFN-τ, the dose of 50 ng/ml that we chose to conduct subsequent experiments is low. For example, uterine infusions of up to 400 μg/day are necessary for extension of estrous cycle length in non-pregnant cows

(Meyer et al., 1995) and others have used doses of 100 ng/ml (Xiao et al., 1999) and up to 20 μg/ml (Asselin et al., 1998) of bIFN–τ for in vitro studies utilizing endometrial epithelial cells. Our data show that bIFN–τ is able to stimulate tyrosine phosphorylation of STAT-1, but not STAT-2 in a dose dependent manner (Figure 5-7). Perhaps if doses between 0 and 3.125 ng/ml of bIFN–τ had been used, a more gradual, dose dependent tyrosine phosphorylation of STAT-2 also would have been observed. Therefore we concluded that 50 ng/ml was adequate for the subsequent studies conducted. This dose effectively suppressed basal and oxytocin-induced $PGF_{2\alpha}$ secretion in primary culture of endometrial epithelial cells (Danet-Desnoyers et al., 1994).

As in typical signal transduction paradigms mediated through tyrosine phosphorylation, bIFN–τ stimulates transient phosphorylation of STAT proteins in WCE (Sillvennoinen et al., 1993; Figures 5-8 and 5-9). The dynamics of phosphorylation indicate the presence of a pool of STAT proteins readily available for phosphorylation. However, after a period of maximum phosphorylation (between 8 and 15 minutes) STATs become refractory to further bIFN-τ-induced tyrosine phosphorylation, which declines. Decreased phosphorylation of STATs after exposure to bIFN–τ longer that 15 minutes may reflect degradation of STATs (Kim and Maniatis, 1996) or dephosphorylation of STATs through the actions of phosphatases (David et al., 1993; Haque et al., 1995). Since abundance of STATs is unchanged over time, the later hypothesis

better explains the data. This agrees with data from Haspel and coworkers (1996), where they followed ^{35}S-labeled STAT-1 throughout an IFN-γ treatment cycle of 4 hours. Only about 10% of STATs were degraded, although 20 to 30% of STATs were in the nucleus by 20 minutes of treatment. Recent reports have identified tyrosine phosphatases from the families of SOCS and PIAS molecules as negative regulators of the JAK-STAT pathway (Chung et al., 1997; Sung and Shuai, 1998; Starr and Hilton, 1999).

Our preliminary experiments with endometrial explants indicate that STATs 1 and 2 (Chapter 4) are present and become phosphorylated in response to bIFN-τ treatment. This indicates that STAT phosphorylation is not an artifact from BEND cells, but a real mechanism occurring in the endometrium.

Among the STATs analyzed, there seemed to be a lower abundance of STAT-2 compared to STAT-1 and -3. This could indicate that STAT-2 is present in lower amounts in cells, or, could be due to an artifact in the methodology used for detection. For example, there could be a lesser affinity of anti-STAT-2 antibody for STAT-2 than the affinity of anti-STATs-1 and -3 antibodies for their respective antigens. There is little evidence for less affinity on immunoblots, since phosphorylation of tyrosine is also less for STAT-2 compared to other STATs. There could be, however, a reduced affinity during immunoprecipitation, which would in turn result in less detectable STAT-2 in immunoblots.

Binding of type-I IFNs to a membrane receptor causes sequential tyrosine phosphorylation of associated JAK kinases and the cytoplasmic tail of the

receptor chains (Ihle et al., 1995; Figure 5-23). Phosphorylation of the receptor attracts unphosphorylated STAT proteins, present in the cytosol, to come in contact with the receptor, where STATs become phosphorylated on tyrosine residues (Greenlund et al., 1994). Phosphorylated STATs form homo- and heterodimers and translocate to the nucleus (Shuai et al., 1994; Schindler and Darnell, 1995). In the nucleus, STAT complexes associate with DNA-binding proteins, such as p48, to form a transcription activation complex which binds to specific response elements on IFN-regulated genes (Darnell et al., 1994; Schindler and Darnell, 1995). Response elements can be for example the interferon-stimulus response element (ISRE, Levy et al., 1988) and the sis-inducible element (SIE, Sadowsky et al., 1993). Our working hypothesis is that proteins synthesized in response to bIFN–τ act to decrease secretion of $PGF_{2\alpha}$. If bIFN–τ-induced or -repressed protein synthesis is a result of activation of the classical JAK-STAT pathway, translocation of tyrosine -phosphorylated STAT proteins to the nucleus is expected. Data in the present report support this possibility.

Generally, bIFN–τ induced an increase in abundance of STAT proteins in the nucleus over time, which reached a plateau and then decreased. Except for numerical differences for STAT-2, this occurred without reciprocal changes in abundance of STATs in CE. This could indicate that only a small fraction of STATs present in the cytosol actually migrated to the nucleus and the immunoblot technique used was not sensitive enough to detect a decrease of

STATs in the CE. Other reports support the concept that STAT molecules cycle to the nucleus as tyrosine phosphorylated molecules, reaching a maximum at 20 to 30 minutes, and later return quantitatively to the cytoplasm as non-phosphorylated molecules (Haspel et al., 1996)

Phosphorylation of STAT-1 increased and peaked sooner in CE than in NE, suggesting a delayed translocation of STAT-1, initially phosphorylated in the cytoplasm, to the nuclear compartment (Figures 5-12 and 5-13). The elevated phosphorylation in control extracts (0 minutes bIFN–τ) is due to a high background that was consistently found in those samples. Although there was no distinguishable band at the region corresponding to the MW of STAT-1, high pixel reading accounted for the elevated densitometric units. These findings are also true for other STATs. After maximum phosphorylation of STAT-1 at 30 minutes in the nucleus there was a steep decrease in phosphorylation observed at 60 minutes (Figure 5-13). During the same time frame, abundance of STAT-1 in the nucleus decreased proportionally less (Figure 5-11). This indicates that dephosphorylation of STAT-1 occurs while the protein is still in the nucleus. This concept is supported by the work of David and others (1993). These authors identified a nuclear tyrosine phosphatase, which deactivated interferon-regulated STATs and down-regulated transcription of interferon-activated genes.

The faster increase and decrease in phosphorylation of STAT-2 in CE compared to STAT-1 suggests a more acute regulation (i.e., phosphorylation and dephosphorylation; Figure 5-12) of this protein. Phosphorylation of STAT-2 in

NE is inconclusive (Figure 5-13 represents the only experiment where phosphorylation of nuclear STAT-2 was detected). However, there is an indication of increased phosphorylation after 15 minutes. Moreover, there is clear increase in abundance of STAT-2 in the nucleus, which occurs earlier than maximum appearance of STAT-1, indicating earlier nuclear translocation (Figure 5-11). Similar to STAT-1 and -3, abundance of STAT-2 in NE was still higher at 120 minutes compared to 0 minutes. This suggests that STATs could be continuously transported to the nucleus, even after 120 minutes. This is consistent with data from Haspel and others (1996) which showed a continuous influx of STAT-1 to the nucleus up to 2.5 hours after interferon treatment.

In contrast to STATs 1 and 2, changes in phosphorylation of STAT-3 in the CE (Figure 5-16) and NE (Figure 5-17) were almost parallel, indicating a ready translocation of phosphorylated STAT-3 to the nuclear compartment. Similar to STAT-1, there is an abrupt decrease in phosphorylation between 30 and 60 minutes in both the CE and NE, but such a decrease is more accentuated in the CE. Since this decrease is not followed by a concomitant change in abundance of STAT-3 (Figure 5-14), it suggests a faster removal of phosphorylated STAT-3 in the CE compared to the NE.

In the coimmunoprecipitation experiments, there was an overall trend of reciprocal changes in abundance of STATs in NE compared to CE (Figures 5-19 to 5-22). Therefore, an increase in abundance of STATs in NE was usually paralleled by a decrease in the CE, and abundance in the CE returned to control

levels by 120 minutes. This indicates that homo and heterodimers of STATs were formed in the cytoplasm, migrated to the nucleus and returned to the cytoplasm.

Association of STATs was noted even without exposure to bIFN–τ, both in NE and CE, indicating a hormone-independent basal level of association of STATs. In support to our findings, Stancato and coworkers (1996) showed that STAT heterodimers (1:2 and 1:3) exist in the cytosol prior to cytokine stimulation. There was a faster rate of increase in abundance of STAT-2 in the nucleus compared to STAT-1, associated with STAT-3. This is in conflict with data from Horvath and Darnell (unpublished observations cited in Darnell, 1997) which suggest weak binding of STAT dimers 2:3, compared to 1:2 or 1:3. However, Ghislain and Fish (1996) reported formation of STAT 2:3 complex after stimulation of U266 cells with interferon-α. Moreover, after maximum abundance at 15 minutes, there was a sharp decrease of STAT-2 in NE, while STAT-1 was still associated with STAT-3 in high amounts by 30 minutes. It is proposed that the dynamics of association with STAT-3 is different for STATs 1 and 2. Furthermore, due to the apparently greater abundance of STAT-1 in cells compared to STAT-2, one should expect greater formation of STAT 1:3 complexes, rather that 2:3. Since the opposite is the case, bIFN–τ apparently preferentially induced formation of STAT 2:3 complexes.

The present series of experiments supports the concept that bIFN–τ is able to activate the JAK-STAT pathway of signal transduction in bovine

endometrial epithelial cells. Complexes of phosphorylated STATs may act as transcription factors to activate transcription of early response genes. Nuclear extracts obtained from BEND cells stimulated with bIFN–τ have factors able to bind ISREs in mobility shift assays, and treatment with bIFN-τ induces synthesis of the interferon regulatory factor 1 (IRF-1; Chapter 6). This suggests that activated STATs are functional and can stimulate gene transcription. Moreover, treatment of BEND cells with bIFN-τ inhibits the phorbol ester-induced stimulation of $PGF_{2\alpha}$ (Chapter 7). Our working hypothesis is that bIFN–τ-stimulated proteins act on endometrial epithelial cells to decrease secretion of $PGF_{2\alpha}$ (Figures 2-3 and 5-23).

One way to confirm that bIFN-τ-induced phosphorylated STAT complexes present in the nucleus are biologically active is to test the ability of proteins in nuclear extracts to bind to specific cis-acting response elements in electrophoretic mobility shift assays. Chapter 6 tests this concept and also examines whether bIFN-τ has the ability to stimulate IRF-1 in BEND cells.

CHAPTER 6
BOVINE INTERFERON-TAU STIMULATES BINDING OF STAT PROTEIN COMPLEXES TO DNA AND STIMULATES SYNTHESIS OF INTERFERON RESPONSE FACTOR-1 (IRF-1) PROTEIN IN BOVINE ENDOMETRIAL (BEND) CELLS

Introduction

It is clear from experimental responses of Chapter 5 that bovine interferon-tau (bIFN-τ) stimulates tyrosine phosphorylation, formation and nuclear translocation of multi-STAT complexes. Type I interferons stimulate gene transcription via a classical mechanism (i.e., JAK-STAT pathway) that includes binding of nuclear STAT complexes to specific sequences in the regulatory region of interferon-regulated genes (Darnell et al., 1994). Sequences include the interferon stimulus response element (ISRE, Levy et al., 1988) and the sis-inducible element (SIE, Sadowsky et al., 1993). Binding of STATs to ISRE and SIE stimulates transcription of genes such as ISG54, ISG15, 6-16, 9-27 and c-Fos (Darnell et al., 1994).

Our working hypothesis is that bIFN-τ-stimulated proteins act to inhibit one or several steps of the cascade for synthesis of $PGF_{2\alpha}$ in the endometrium (Figure 2-3). Type I interferons direct transcription of both IRF-1 and IRF-2 (Harada et al., 1989), and IRF-1 directly activates expression of IRF-2

(Henderson et al., 1997). Conversely, IRF-2 negatively regulates expression of IRF-1, characterizing a yin-yang type of regulatory paradigm (Harada et al., 1989, 1994).

IRF-2 is a protein induced by ovine IFN-τ in the endometrium of sheep (Spencer et al., 1998). IRF-2 is a transcription repressor that inhibits transcription of genes such as EBNA-1 (Zhang and Pagano, 1999) and NOS-2 (Faure et al., 1997). It is possible that, in the endometrium, IRF-2 suppresses synthesis of proteins critical to the generation of $PGF_{2\alpha}$. In fact, sequences of regulatory regions of the bovine oxytocin receptor (Bathgate et al., 1998) and the ovine estrogen receptor (Fleming et al., 1998) genes have putative sequences for binding to members of the IRF family (IRF response elements, or IREs).

Our objectives were (1) to characterize binding of nuclear proteins from b-IFN-τ stimulated BEND cells to ISRE and SIE elements, (2) identify nature of nuclear proteins binding ISRE and SIE elements and (3) to examine ability of bIFN-τ to induce synthesis of IRF-1 in BEND cells.

Materials and Methods

Materials

Bio-Rad protein assay, TEMED and ammonium persulphate were from Bio-Rad Laboratories (Hercules, CA). Bovine IFN-τ (dissolved in 20 mM Tris-HCl, 1 mM EDTA, pH 8 to 200 μg/ml; 1.08×10^7 units of antiviral activity) was a

generous gift from Dr. Michael Roberts (University of Missouri). Complementary oligodeoxyribonucleotides corresponding to the interferon stimulus response element (ISRE; double stranded, 100 ng/μl solution) and to the sis-inducible element (SIE; sense and anti-sense strands, 100 ng/μl solutions) were obtained as a gift from Dr. Douglas Leaman (The Cleveland Clinic Foundation, OH). Sequences of the sense (5' to 3') strands were as follows: ISRE, 5'-TTTACAAACAGCAGGAAATAGAAACTTAAGAGAAATACA-3', from the 9-27 gene; SIE, 5'-AGCTTCATTTCCCGTAAATCCCTA-3', from the c-Fos gene. T4 Polynucleotide kinase (100 units in a 10 units/μl solution; supplied with 10 X kinase reaction buffer), Klenow kit (contains 150 units Klenow in a 5 units/μl solution, 10 X polymerase reaction buffer and 500 μl each 10 mM dATP, dCTP, d GTP, dTTP) herring sperm DNA were from Promega Corp (Madison, WI). Gamma ^{32}P-ATP (5 mCi in 30 μl; specific activity: 7000 Ci/mMol) and alpha ^{32}P-dCTP (1 mCi in 100 μl; specific activity: 3000 Ci/mMol) were from ICN (Costa Mesa, CA). Tris, EDTA, phenol, borosilicate Pasteur pipettes (5.75"), ammonium acetate, tris-HCl, KCl, $MgCl_2$, DTT, glycerol, xylene cyanol and Whatman® filter paper were purchased from Fisher Scientific (Pittsbugh, PA). Sephadex G-10 beads, rabbit IgG and tRNA were from Sigma Chemical Co. (St. Louis, MO). Acrylamide and NN'-methylenebisacrylamide were from BDH Laboratory Supplies (Poole, England). Poly dIdC (10 A_{260} units diluted in 400 μl of sterile water) and ATP were from Boehringer Mannheim Co. (Indianapolis, IN). X-ray

films were from NEN Research Products (Reflection; Boston, MA) and from Eastman Kodak Co. (X-Omat Blue XB-1; Rochester, NY). Konica -X-ray film processor model QX-70 was from Konica Corp.(Japan). Centricons (10.000 MWCO) were from Amicon Inc. (Beverly, MA). Anti-IRF-1 polyclonal antibody (C-20, catalog number SC-497X, 200μg/100μl), anti-STAT-1 (E-23; catalog number SC-346X, 200μg/100μl) and anti-STAT-2 (C-20; catalog number SC-476X, 200μg/100μl), anti-STAT-3 (C-20; catalog number SC-482, 200μg/1ml) were from Santa Cruz Biotechnology (Santa Cruz, CA).

Probes

For annealing, sense and anti-sense strands (25 μl each) of SIE were mixed in a 500 μl microfuge tube and placed in a beaker containing water at 100 C. The beaker was removed from heat, and water allowed to cool to room temperature. Annealed probes were stored at -20 C. Annealed, complementary oligodeoxyribonucleotides corresponding to ISRE and to SIE were labeled radioactively for experiments. Probes for ISRE were prepared by end labeling. Two hundred ng of double stranded DNA (2 μl of stock solution) were mixed with 5 μl of 10X polynucleotide kinase buffer, 40 μl of water, 2 μl of gamma ^{32}P-ATP (333 μCi) and 1μl T4 polynucleotide kinase in a microfuge tube (500μl) and incubated for 30 minutes at 37 C. Then, 5μl of 250 mM ATP were added to the reaction mixture, and incubated for 10 minutes at 37 C. Finally, 10 μl of tRNA (1

mg/ml in water) and 5 µl of phenol saturated in 10 mM Tris 1 mM EDTA pH 8 buffer (TE) were added. The mixture was loaded onto a Sephadex-G10 chromatographic column. The chromatographic column was prepared to purify fractions containing radiolabeled probe from the remaining reaction mixture. A disposable borosilicate Pasteur pipette (5.75") was secured in a ring stand with a clamp and used as a column. Autoclaved glass wool was packed at the bottom of the column and moistened with TE buffer. A slurry of Sephadex G-10 beads (50 % beads:50%TE buffer, prepared previously and kept at 4C) was added to the column until beads were packed to approximately 5 mm above the ridge of the column. The TE buffer was added continuously to the top of the column to prevent beads from drying. When the labeling mixture was ready, the column was allowed to run dry, and reaction mixture was loaded immediately onto the column. Then, while adding TE buffer to the column in 100 µl volumes, 100 µl fractions were collected into microcentrifuge tubes. Fractions were monitored for radioactivity using a portable geiger counter and the three most active fractions were pooled. Five µl of tRNA solution, 30 µl of 8 M ammonium acetate (kept at -20 C) and 700 µl 100% ethanol (Kept at -20 C) were added to the combined fractions. This entire mixture was hand mixed and incubated at -80 C for 2 hours. Then, mixture was thawed, centrifuged at 12000 x g for 15 minutes, supernatant carefully aspirated and discarded. Pellet (not visible) was washed in 200 µl 70% ethanol (kept at -20 C), centrifuged at 12000 x g for 5 minutes, supernatant removed and discarded. Remaining ethanol was removed by

placing the tube in a speed vacuum apparatus for 10 minutes at room temperature. Dried pellet was resuspended in 300 μl TE (hereafter referred to as "labeled probe"). Two μl of labeled probe were measured in duplicate to determine specific activity. Specific activity of labeled probe was 140000 cpm/μl. Radiolabeled probe was stored at -20 C until use in assay for electrophoretic mobility shift assays (EMSA).

Probes for SIE were prepared by fill-in. A dNTP mix was prepared mixing 10 μl each of 10 mM dATP, dTTP and dGTP, resulting in a mix containing 3.33 mM of each deoxyribonucleotide. Two hundred ng of double stranded (i.e., annealed) SIE DNA (2 μl of stock solution) were mixed with 4μl of 10X Klenow polymerase buffer, 16μl of water, 6 μl of dNTP mix, 10 μl of alpha ^{32}P-dCTP (100 μCi) and 2 μl of Klenow in a 500 μl microfuge tube and incubated for 30 minutes at 37 C. The remainder of the procedure was as described for labeling of the ISRE probe. Specific activity of labeled probe was 42000 cpm/μl. Labeled probe was stored at -20 C until used for EMSA.

Electrophoretic Mobility Shift Assays

General. A 5X EMSA buffer (10 mM Tris -HCl pH 7.4, 60 mM KCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 DTT, 10% glycerol, 4 μg herring sperm DNA) was prepared and stored at -20 C. Ten fold excess cold probes were prepared as follows: based on volume of labeled probe added per tube, mass of DNA present

was estimated, and a 10 fold excess of unlabeled probe solution was prepared by diluting appropriate oligodeoxyribonucleotides (i.e., ISRE or SIE) in water. Six % acrylamide, large format gels were set up and allowed to polymerize overnight. Samples were prepared in 500 µl microfuge tubes, and included appropriate nuclear extracts (NE), 8µl 5X EMSA buffer, 2µl poly dIdC and sterile water brought up to a final volume of 40µl. When required, a 10 fold excess of unlabeled probe, specific antibodies (1 µl each of anti STAT-1, anti-STAT-2 or anti-IRF-1 or 10 µl of anti-STAT-3) or rabbit IgG (1 µl of a 200 µg/ml solution) were added. Volume of water was calculated so that final volume of the reaction mix was 40 µl. This mix was incubated at room temperature (Experiment 1) or at 37 C (Experiments 1, 2 and 3) for 30 minutes (pre-incubation period). Subsequently, specific labeled probe was added to the reaction mix (100000 cpm) and incubated at 37 C for a 30 minute binding period (ISRE, Experiments 1 and 2; SIE, Experiment 3). During pre-incubation and binding periods, gels were pre-electrophoresed, at maximum voltage and constant 30 mA current per gel. Five µl of gel dye (40% glycerol, 60% water, 0.01% xylene cyanol) were added in each tube, mixed and loaded onto gel. Gels were run for 5 to 6 hours at constant 30 mA. After electrophoresis, a sheet of Whatman filter paper was placed in contact with surface of gel, and gel was relocated from glass plate to adhere to filter paper. Gel was covered with plastic wrap and dried on a on a slab gel drier (model SE 1150, Hoeffer Scientific Instruments, San Francisco,

CA; 50 minutes at 80 C). Dried gels were exposed to X-ray films overnight (all experiments) and for 7 hours (Experiment 3) at -80 C and developed in a Konica -X-ray film processor model QX-70 (Konica Corp., Japan).

Experimental designs. Experiment 1 was designed to define ideal experimental conditions to be used in subsequent experiments. Two pre-incubation/binding temperatures (room temperature vs. 37 C) and two amounts of nuclear protein (10 vs. 20 μg) were tested for nuclear extracts obtained from BEND cells treated in presence or absence of bIFN-τ. Experiment 2 was designed to identify nature of proteins contained in complexes induced by bIFN-τ and bound to an ISRE labeled probe, through use of specific antibodies against STATs 1, 2 and 3 and against IRF-1. Specificity of antibody action was verified incubating a sample with rabbit IgG. Protein from nuclear extracts (10 μg) was used in each pre-incubation reaction. Pre-incubation and binding reactions were carried out at 37 C. Experiment 3 was designed to identify nature of proteins contained in complexes induced by bIFN-τ, and bound to an SIE labeled probe, through use of specific antibodies against STATs 1 and 3. Specificity of antibody action was verified incubating a sample with rabbit IgG. Protein from nuclear extracts (7 μg) was used in each pre-incubation reaction. Pre-incubation and binding reactions were carried out at 37 C.

Immunoblotting for IRF-1

To verify whether bIFN-τ was able to induce synthesis of the early gene IRF-1, BEND cells were grown to 90% confluency as described in Chapter 5. Then, cells were washed (20 ml) and incubated (19 ml) in serum-free medium for 1 hour. Nuclear extracts were obtained as described for Chapter 5 from one plate immediately after this 1 hour starvation, and from the remaining eight plates after they received 0 or 50 ng/ml bIFN-τ for 1, 2, 4 or 6 hours. Treatments were diluted in 1 ml of medium before adding to plates (final volume = 20 ml/per plate). Hypotonic buffer (see Chapter 5) was added to NE to a final volume of 2 ml, and adjusted to 60 mM KCl adding 1 M KCl as needed. Diluted NEs were then concentrated using 10000 molecular weight cutoff Centricons overnight, in a Sorvall RC-5B refrigerated superspeed centrifuge, equipped with a SM24 (Du Pont Co., Wilmington, DE) at 5000 RPM. Retentates (approximately 50 μl) were recovered and analyzed for protein content using the Bradford method (Bio-Rad protein assay). Volumes corresponding to 40 μg of total protein were solubilized in 2X Laemmli buffer (25 μl) and water (up to 50 μl final volume) and loaded onto 7.5% acrylamide mini-gels. Transfer to nitrocellulose membrane and immunoblotting were performed as described in Chapter 5. Briefly, membrane was blocked in 5% non-fat dried milk in TBST for 2 hours, washed, incubated with anti-IRF-1 polyclonal antibody (2 mg/ml; 1:10000) diluted in 5% non-fat dried milk in TBS for 2 hours, washed, incubated with anti-rabbit-IgG secondary

antibody (1:8000) for 1 hour, washed and proteins detected using an enhanced chemiluminescence kit, with exposures of 3 minutes. Immunoblots for IRF-1 were analyzed by densitometry as described in Chapter 5.

Statistical Analysis

Abundance of IRF-1 also was analyzed by least squares analysis of variance using the GLM procedure of SAS (SAS, 1988). Independent variables were gel, treatment, time, gel by treatment, gel by time and treatment by time. Since there was only a negligible signal for IRF-1 in the absence of bIFN-τ, data were re-analyzed for the effect of time on samples treated with bIFN-τ only.

Results

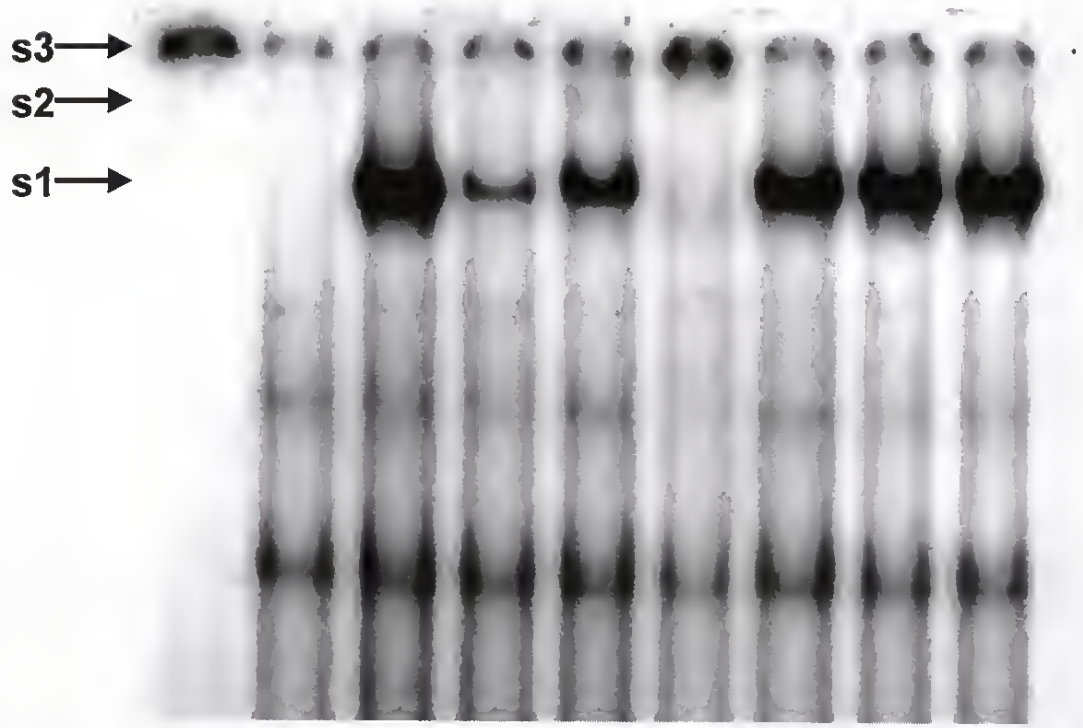
Electrophoretic Mobility Shift Assays (EMSA)

Experiment 1 indicated that incubation with NE from cells treated with bIFN-τ produced slower migrating bands (shifted complexes;s1) compared to incubation with NE from untreated cells (Figure 6-1). Reactions performed at 37 C produced stronger bands than room temperature regardless of amount of NE protein used. Intensity of shifted complexes was reduced greatly when a 10-fold excess of non-radioactively labeled probe was added to the incubation reactions. Experiment 2 revealed that no specific complexes were formed in absence of nuclear extracts (lane 1; Figure 6-2) or when nuclear extracts obtained from

bIFN-τ	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
temp[a]	rt	rt	rt	rt	37	37	37	rt	rt	rt	rt	37	37	37	37
μg prot[b]	10	20	10	20	10	20	20	10	20	10	20	20	10	10	20
10x cold[c]	-	-	+	+	-	-	+	-	-	+	+	-	-	+	+

Figure 6-1. Experiment 1. Electrophoretic mobility shift assay of nuclear extracts of BEND cells incubated with radiolabeled ISRE in presence (+) or absence (-) of bIFN-τ for 30 min. Arrow indicates a shifted complex (s1).
[a] Temperature of reaction (rt=room temperature; 37=37 C).
[b] Amount of protein loaded in μg.
[c] Presence (+) or absence (-) of 10 fold excess ISRE unlabeld probe.

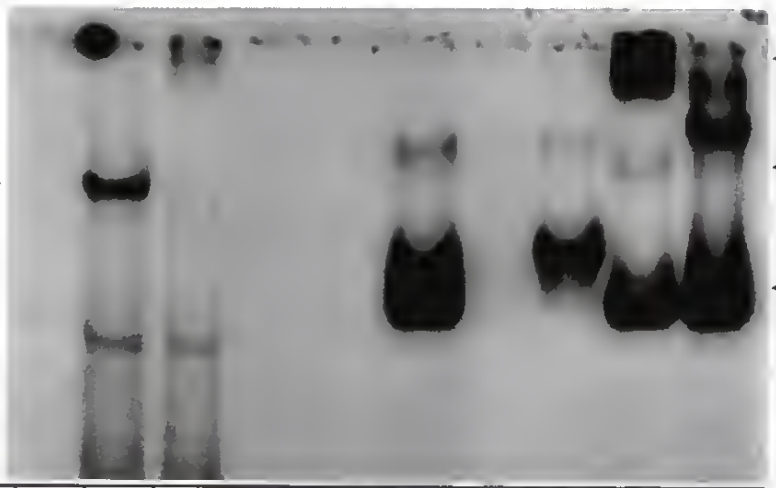
untreated cells were used (lane 2). The apparent s3 complex present in lane 1 is non-specific, and was not observed in a subsequent experiment (Figure 6-3). Formation of s1 complex was noticed when nuclear extracts from cells treated with bIFN-τ were used (lane 3), and intensity of such complex was reduced when a 10 fold excess cold ISRE probe was added to the reaction mixture (lane 4). Addition of an anti-STAT-1 antibody caused a reduction in intensity of s1, which may be associated with the formation of the low-intensity s2 complex. Anti-STAT-2 antibody abolished formation of s1 complex, but stimulated formation of a s3 complex, which was not resolved in the present gel. Formation of an anti-STAT-2-induced s3 complex was confirmed in a subsequent experiment (Figure



NE[a]	-	0	30	30	30	30	30	30	30
10 x cold[b]	-	-	-	+	-	-	-	-	-
anti-STAT-1	-	-	-	-	+	-	-	-	-
anti-STAT-2	-	-	-	-	-	+	-	-	-
anti-STAT-3	-	-	-	-	-	-	+	-	-
rabbit IgG	-	-	-	-	-	-	-	+	-
anti-IRF-1	-	-	-	-	-	-	-	-	+

Figure 6-2. Experiment 2. Electrophoretic mobility shift assay of nuclear extracts of BEND cells incubated with radiolabeled ISRE. Ingredients listed in the first column were present (+) or absent (-) in pre-incubation reaction (see text). Arrows indicate shifted complexes (s1, s2, s3).
[a] Nuclear extract used: (-) none, (0) from BEND cells incubated in medium alone for 30 minutes, (30) from BEND cells incubated with bIFN-τ for 30 minutes
[b] Presence (+) or absence (-) of 10 fold excess ISRE unlabeld probe.

a



ISRE	+	+	+	-	-	-	-	-	-	-
SIE	-	-	-	+	+	+	+	+	+	+
NE[1]	-	30	30	-	0	30	30	30	30	30
10 x cold SIE	-	-	-	-	-	-	+	-	-	-
anti-STAT-1	-	-	-	-	-	-	-	+	-	-
anti-STAT-2	-	-	+	-	-	-	-	-	-	-
anti-STAT-3	-	-	-	-	-	-	-	-	+	-
rabbit IgG	-	-	-	-	-	-	-	-	-	+

b

Figure 6-3. Experiment 3. Electrophoretic mobility shift assay of nuclear extracts of BEND cells incubated with radiolabeled ISRE or SIE.

a) Ingredients listed in the first column were present (+) or absent (- ; see text). Arrows indicate shifted complexes (s1, s4, s5, s6); b) Detail of panel a obtained at a lower exposure time. Partition of complex s4 into 3 sub-complexes, s4a, s4b, s4c.

[1] Nuclear extract used: (-) none, (0) from BEND cells incubated in medium alone for 30 minutes, (30) from BEND cells incubated with bIFN-τ for 30 minutes

[2] Presence (+) or absence (-) of 10 fold excess SIE unlabeld probe.

6-3, lanes 1 to 3). Anti-STAT-3, anti-IRF-1 and rabbit IgG had no effect on migration or intensity of s1 complex.

In Experiment 3, no specific complexes were formed in the absence of nuclear extracts (lane 4; Figure 6-3) nor when nuclear extract from untreated cells was used (lane 5). Treatment with bIFN-τ induced formation of a major and a minor complex (s4 and s5 respectively; lane 6). Formations of both s4 and s5 complexes were abolished completely when a 10-fold excess of unlabeled SIE probe was added (lane 7). Addition of antibodies against STATs 1 and 3 revealed that s4 was composed of three distinct sub-complexes (Figure 6-3, panel b), a faster (s4c), an intermediate (s4b) and a slower (s4a) migrating complexes. Presence of anti-STAT-1 antibody displaced both s4b and s4c, but intensity of s4a was actually enhanced. Pre-incubation with anti-STAT-3 caused displacement of s4a and s4b to form the "supershifted" complex s6. In contrast, s4c migration remained unaltered. The fact that s4b was displaced both by anti-STAT-1 and -3 antibodies, suggests that complex contains both STATs, and is probably a STAT 1:3 heterodimer. Since s4c was displaced when anti-STAT-1 antibody was used, but not anti-STAT-3 suggests it represents a STAT-1:1 homodimer. Similarly, s4a displacement with anti-STAT-3 exclusively suggests that it is composed of STAT 3:3 homodimers. The s5 was formed of two sub-complexes that had similar alterations on migrating pattern to s4 sub-complexes in response to STAT-1 and STAT-3 antibodies. Addition of rabbit IgG did not alter intensity or mobility of s4, but may have affected migration properties of s5.

Immunoblotting for IRF-1

There was a significant time by treatment interaction on abundance of IRF-1 (Figure 6-4; $p<0.03$). Treatment with bIFN-τ, but not with medium alone, stimulated synthesis of IRF-1 in a time dependent manner ($p<0.04$). IRF-1 increased after 1 hour of treatment to reach a maximum at 2 hours and gradually decreased after 4 and 6 hours of exposure to bIFN-τ.

Discussion

When BEND cells were stimulated with bIFN-τ, nuclear proteins bound to specific response elements (i.e., ISRE and SIE) present in the regulatory region of genes stimulated by interferons. Moreover, synthesis of the transcription factor IRF-1 was documented. These findings support the concept that the JAK-STAT signal transduction system described in Chapters 4 and 5 is indeed functional in BEND cells. Data from Experiment 2 indicated clearly the formation of a specific complex (s1) when nuclear extracts from bIFN-τ-treated cells were used. The radiolabeled ISRE probe is from the 9-27 interferon-induced gene, and contains the classical ISRE sequence of nucleotides (Parrington et al., 1993; Darnell et al., 1994). Therefore, complex s1 probably is comprised of the ISGF-3 proteins: STAT-1, STAT-2 and p48 (Shindler et al., 1992; Darnell et al., 1994), which is known to bind to such a sequence. Our attempt to elicit a supershift of this complex with antibodies for STATs 1 and 2 yielded mixed results. Antibody

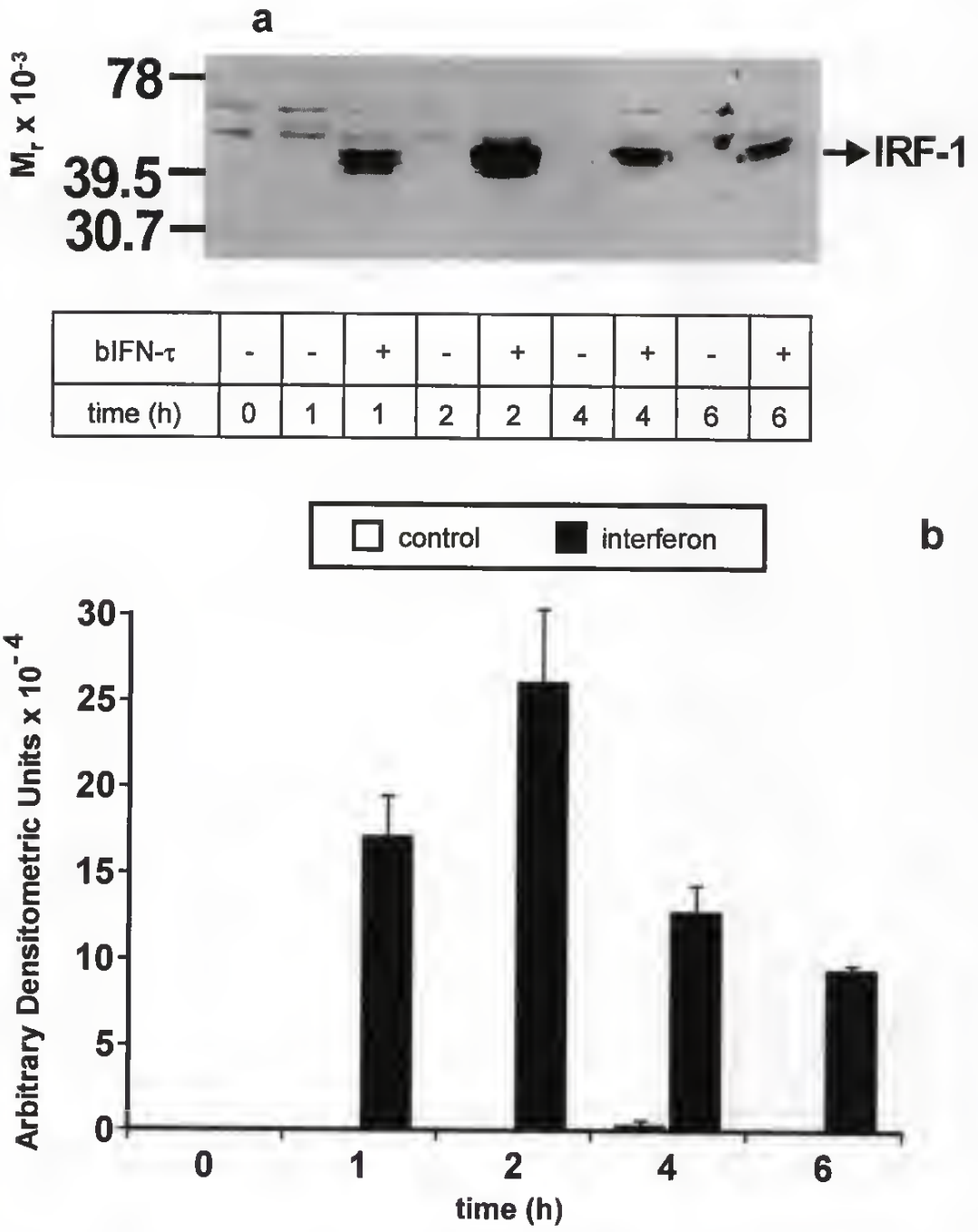


Figure 6-4. Immunoblotting analysis of IRF-1 in nuclear extracts from BEND cells incubated in presence (+) or absence (-) of bIFN-τ (50 ng/ml) for increasing intervals of time.

a) Representative enhanced chemiluminescence exposure of abundance of IRF-1; b) Densitometric analysis of abundance of IRF-1 (least squares means±SEM) .

for STAT-1 elicited formation of the s2 complex, but band was faint and its significance must be further confirmed. Experiments with alternative conditions during the pre-incubation period may yield more definitive results (e.g., different temperatures). In contrast, antibody for STAT-2 caused complete displacement of s1. This could be due to two reasons. First, binding of STAT-2 antibody to STAT-2 on the ISGF-3 complex may cause conformation changes that prevent appropriate binding of ISGF-3 to the ISRE probe. A second possibility is that the a supershifted complex was formed, but became too bulky to be resolved in a 6% acrylamide gel. In fact, other reports demonstrating supershifted ISGF-3 complexes used lower percentage acrylamide gels (4.5%; Ghislain and Fish, 1996; 5%; Yang et al., 1996). This preferred alternative is substantiated by the formation of s3 complex (lane 6, Figure 6-2). Since a s3-like complex also was formed in the absence of NE (lane 1, Figure 6-2), one could argue that s3 is non-specific. A follow up experiment demonstrated that formation of s3-like complex in the absence of NE in Experiment 2 was probably an artifact, because no such complex was present in the absence of NE, but was present in NE pre-incubated with anti-STAT-2 (Figure 6-3). Lack of alteration on s1 by anti-STAT-3 antibody or rabbit IgG further confirmed specificity of the ISRE probe to the ISGF-3 complex that does not contain STAT-3. Absence of supershift in response to anti-STAT-3 also was noted by Yang and coauthors in Daudi cells (1996). IRF-1 can stimulate transcription of the 9-27 interferon-inducible gene through binding at the ISRE of that gene (Henderson et al., 1997). This was not noted in the

present experiment, since anti-IRF-1 caused no supershift. This probably occurred because NE used in the present study were from cells treated with bIFN-τ for only 30 minutes, which is not enough time to allow synthesis of sufficient amounts of IRF-1 to bind to ISRE (based on Figure 6-4, maximal protein expression of IRF-1 was not noted until 2-hour exposure to bIFN-τ).

In Experiment 3, bIFN-τ also induced formation of two specific complexes, s4 and s5 (SIE probe). However, nature of such complexes were probably different than those observed in Experiment 2 (ISRE probe). The SIE sequence is present in the c-fos gene and shown to be regulated by binding of STAT-1 (Fu and Zhang, 1993) and STAT-3 (Rajotte et al., 1996) homo-and heterodimers. Indeed, Yang and coauthors (1996) reported binding of STAT-1 and -3, but not STAT-2 to SIE labeled probes after incubations with NE from interferon-treated cells. In Experiment 3, pre-incubation with anti-STAT-1 and anti-STAT-3 antibodies yielded formation of distinct supershifted complexes, indicating that both proteins are present and have the ability to bind specific DNA sequences. The supershifted complexes were similar to those reported by Yang and others (1996). Pre-incubation with anti-STAT-1 antibody caused supershift of the faster migrating s4c sub-complex, but the supershifted subcomplex could not be visualized in the gel. Examination of data in Yang and others (1996) shows that the supershifted sub-complex elicited by pre-incubation with STAT-1 antibody is slower migrating than the supershifted sub-complex formed by pre-incubation with STAT-3 antibody. Since the supershifted sub-complex elicited by STAT-3

was located at the very top of the EMSA gel, it is presumed that the STAT-1 antibody supershifted sub-complex was of higher molecular weight and therefore did not enter the gel. Alternatively, STAT-1 antibody may have interfered with binding of STAT-1-containing complexes (i.e., STAT 1:1 and STAT 1:3) to the SIE probe. This would explain why s4a was more intense when anti-STAT-1 was added to the pre-incubation mixture (lane 8) compared to lane 6. Since binding of STAT complexes to the labeled SIE is probably competitive, the s4a complex, probably formed by STAT 3:3 homodimer, had a greater chance to bind to the SIE, causing increase in intensity of that sub-complex.

Collectively, EMSA experiments confirmed and expanded findings of Chapters 4 and 5. STAT proteins indeed migrated to the nucleus upon activation with bIFN-τ. More importantly, these activated STATs formed complexes with the ability to bind sequences of DNA contained in interferon-regulated genes. This supports the concept that bIFN-τ stimulates synthesis of proteins through the JAK-STAT pathway. Our working hypothesis is that bIFN-τ-induced proteins act to inhibit the pathway of $PGF_{2\alpha}$ production in the endometrium. In fact, one such protein is the bovine ubiquitin cross-reactive protein (bUCRP; Perry et al., 1997), which promoter region contains five putative ISRE elements. For example, UCRP forms conjugates with cytosolic proteins, possibly altering their cellular activity (Johnson et al., 1998a). Conjugated proteins may include proteins involved in the synthesis of $PGF_{2\alpha}$, such as the

oxytocin receptor, estrogen-receptor, protein kinase C, phospholipase A_2 and/or cyclooxygenase 2.

Another way to influence negatively the $PGF_{2\alpha}$ generation pathway is to stimulate synthesis of transcription repressors. In the ewe, IFN–τ induces synthesis of IRF-2, a transcription repressor (Spencer et al., 1998). Moreover, the promoter region of estrogen receptor gene, which is involved in luteolysis, contains binding sites for IRFs (IRE, Fleming et al., 1998). Furthermore, promoter deletion experiments indicated that treatment with bIFN–τ decreased expression of an estrogen-receptor-luciferase reporter construct, but such a decrease was noted only if the region containing the IRE was present (Fleming et al., 1998). In cattle, the oxytocin receptor also is probably involved in the luteolytic cascade. The oxytocin receptor gene has been sequenced recently and also contains an IRE element, which binds both IRF-1 and IRF-2 (Bathgate et al., 1998). Nuclear extracts from endometrium of pregnant cows can bind to the IRE in mobility shift assays. However, binding can not be supershifted with anti-IRF-1 or -2 antibodies, indicating that other members of the IRF family may be involved (Bathgate et al., 1998).

In the present study, bIFN-τ induced IRF-1 protein expression in BEND cells. Attempts were made to measure induction of IRF-2 after exposures to bIFN-τ ranging from 1 to 48 hours, but failed probably due to use of heterologous reagents (anti-human IRF-2 antibody; data not shown). However, since IRF-2 expression can be induced by IRF-1 (Henderson et al., 1997), it is possible such

a response takes place in bIFN-τ -stimulated BEND cells. Alternatively, IRF-1 could regulate transcription of unrecognized genes with potential effects on the $PGF_{2\alpha}$ generation cascade.

The working hypothesis of this dissertation is that bIFN-τ acts through the JAK-STAT pathway to reduce production of $PGF_{2\alpha}$ in the endometrium. Chapters 4, 5 and 6 demonstrated the presence and functionality of this pathway in BEND cells. Next chapter will examine whether bIFN-τ has the ability to reduce $PGF_{2\alpha}$ in BEND cells.

CHAPTER 7
INTERFERON-TAU MODULATES PHORBOL ESTER-INDUCED SECRETION OF PROSTAGLANDIN AND PROTEIN EXPRESSION OF PHOSPHOLIPASE-A2 AND CYCLOOXYGENASE-2 FROM BOVINE ENDOMETRIAL (BEND) CELLS

Introduction

Both in vivo (Meyer et al., 1995) and in vitro (Helmer et al., 1989; Danet-Desnoyers, 1994; Xiao et al., 1999) experiments demonstrated that bIFN-τ is able to attenuate endometrial secretion of $PGF_{2\alpha}$. A variety of experimental models have been used to demonstrate this effect of bIFN-τ, including explants (Helmer et al., 1989), primary cultures of endometrial epithelial cells collected on days 1 to 4 (Xiao et al., 1999) or 15 of the estrous cycle (Danet-Desnoyers, 1994). The aforementioned approaches involve synchronization and slaughter of animals at specific stages of the estrous cycle or obtaining tissue from the slaughterhouse, in order to collect cells to conduct experiments. These approaches are both time and resource consuming. Moreover, secretion of $PGF_{2\alpha}$ is highly variable among animals, which make interpretation of results challenging. An alternative experimental model for studying effects of bIFN-τ in the endometrium is use of BEND cells. BEND cells are a line of spontaneous replicating endometrial epithelial cells originating from day 14 cyclic cows (Austin

et al., 1996b). Our goal was to characterize the effect of bIFN-τ on secretion of $PGF_{2\alpha}$ from BEND cells, and our working hypothesis was that bIFN-τ should have an inhibitory effect on stimulated $PGF_{2\alpha}$ synthesis.

Oxytocin stimulates secretion of $PGF_{2\alpha}$ from primary endometrial epithelial cells in culture (Danet-Desnoyers et al., 1994). Oxytocin binds to a seven transmembrane-domain, G protein-coupled receptors and activates phospholipase C (PLC). The PLC cleaves membrane phosphotydilinositol bisphosphate, yielding inositol trisphosphate (IP_3) and diacylglycerol (DAG). The IP_3 binds to specific receptors in the endoplasmic reticulum resulting in release of calcium from internal stores into the cytosolic compartment. The DAG activates protein kinase C (PKC), leading to serine phosphorylation of cytosolic, calcium-dependent phospholipase A_2 (PLA_2), probably through a MAP-kinase dependent pathway (Lin et al., 1993). The IP3-stimulated increase in cytosolic calcium acts to further stimulate PLA_2 activity (Clark et al., 1991). Stimulated PLA_2 translocates to the membrane where phospholipid substrates are located (Clark et al., 1991). Activated, membrane-bound PLA_2 cleaves arachidonic acid (AA) from phospholipids. Free AA is converted to prostaglandin H_2 (PGH_2) by the enzyme cyclooxygenase-2 (COX-2). Prostaglandin $F_{2\alpha}$ synthase converts PGH_2 into $PGF_{2\alpha}$, which is then released into the uterine circulation (Smith et al., 1991). However, in a preliminary experiment (Binelli, Arnold and Thatcher, unpublished results) it was determined that one hour treatment with oxytocin failed to stimulate secretion of $PGF_{2\alpha}$ from BEND cells. Furthermore, Arnold and

coauthors (1999) demonstrated that treatment of day 15 endometrial explants with phorbol 12,13 dibutyrate (PDBu; an stimulator of PKC activity), but not with oxytocin, stimulated production of $PGF_{2\alpha}$. Working with endometrial cells collected on days 1 to 4 of the estrous cycle, Xiao and coworkers (1999) determined that phorbol 12-myristate 13-acetate (PMA) stimulated both secretion of $PGF_{2\alpha}$ and expression of COX-2 protein. In addition, these authors reported that bIFN-τ reduced both of these PMA-induced effects. Objectives of the present study were (1) to test whether PDBu could stimulate $PGF_{2\alpha}$ secretion from BEND cells and whether bIFN-τ could block such an effect; (2) to study the time-response dynamics of PDBu-induced $PGF_{2\alpha}$ secretion in presence and absence of bIFN-τ; (3) to examine the effects of PDBu on PLA_2 and COX-2 protein expression in presence and absence of bIFN-τ, and (4) to investigate whether bIFN-τ could inhibit $PGF_{2\alpha}$ secretion in BEND cells previously stimulated with PDBu.

Materials and Methods

Materials

Tissue culture treated, polystyrene Costar® 6 well plates were from Corning Inc. (Corning, NY). The PDBu (diluted to 1 mg /ml in 100% ethanol) and rabbit IgG (diluted to 1 mg/ml in TBS; stored at -20 C) were from Sigma Chemical, St. Louis, MO. Recombinant bIFN-τ (dissolved in 20 mM Tris-HCl, 1

mM EDTA, pH 8 to 200 μg/ml; 1.08 x 10^7 units of antiviral activity) was a generous gift from Dr. Michael Roberts, University of Missouri. Isotopically labeled [5, 6, 8, 11, 12, 14, 15-^{3}H]-$PGF_{2\alpha}$ (specific activity=212 Ci/mole) was from Amersham Corp. (Arlington Heights, IL). Tris-HCl, Tween 20, isopropyl alcohol and chloroform were from Fisher Scientific (Pittsburgh, PA). Anti-COX-2 polyclonal antibody was from Cayman Chemical (Ann Arbor, MI; lyophilized anti-COX-2 antibody was reconstituted in 200 μl sterile water, aliquoted in 20 μl portions and stored at -20 C). Cytosolic PLA_2 polyclonal antibody (N-216; catalog number SC-438, 200μg/ml) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Material used for cell culture, whole cell extracts (WCE) and for immunoblotting procedures were as described for Chapter 5.

Cell Culture and Sample Collection

BEND cells were plated on 100 mm tissue culture-treated petri dishes (8.5 x 10^5 cells/plate; 20 ml of medium, Experiment 1; 10 ml of medium, Experiments 2, and 3) or on 35 mm wells on 6-well plates (2 x 10^5 cells /well; 3 ml of medium, Experiment 4). Cells were grown to confluency, washed in serum-free medium (20 ml, Experiment 1; 10 ml, Experiments 2 and 3; 3 ml Experiment 4) and cultured for 24 hours (same volumes used for washes) in serum-free medium. After this time, a sample of medium was collected and stored at -20 C. This was designated "0" hour sample. Then cells were washed again and serum

free medium was mixed with appropriate treatments and added to cells in the same volumes listed above. Samples of medium (500 μl in Experiment s 1, 2, and 3; 250 μl in Experiment 4) were collected at specified times (see Experimental designs) and stored at -20 C. After sampling, the same volume of medium removed was replaced with medium containing the appropriate treatment, so that a constant volume was maintained throughout each experiment. Concentrations of $PGF_{2\alpha}$ were measured in medium as described below.

Radioimmunoassay

Concentrations of $PGF_{2\alpha}$ were measured in medium undiluted (control-, bIFN-τ- and PDBu + bIFN-τ-treated cells) or diluted 1:2 in medium (PDBu-treated cells). Twenty five μl of each sample were further diluted in 75 μl of 50 mM Tris-HCl, pH 7.5 (Tris buffer). Therefore, total volume of diluted sample for assay was 100 μl. Radioimmunoassay procedure was described by Danet-Desnoyers and coworkers (1994). Assay was validated for serum-free medium adding 25 pg/.1 ml $PGF_{2\alpha}$ to medium. Average recovered $PGF_{2\alpha}$ was 25.1±1.42 pg/.1 ml, which yielded a calculated recovery of 100.25%. To prepare standard curves, known amounts of non-radioactive $PGF_{2\alpha}$ (1.25 to 1000 pg/tube) were diluted in Tris buffer (100 μl). Anti-$PGF_{2\alpha}$ antiserum (characterized by Dubois and Bazer, 1991) was diluted 1:5000 (Tris buffer; 100 μl/tube). Final volume of sample was

400 µl (100 µl Tris buffer, 100 µl diluted sample, 100 µl ab solution, 100 µl radiolabeled $PGF_{2\alpha}$ solution). Minimum detectable concentration of $PGF_{2\alpha}$ was 3.32 pg/tube. Inter- and intra-assay coefficients of variation were 13.99 and 12.61% respectively. Since multiple samples were removed, and medium was added back to plates, concentrations of $PGF_{2\alpha}$ were adjusted to account for $PGF_{2\alpha}$ removed from previous samples. Adjustment consisted on adding the amount of $PGF_{2\alpha}$ removed in previous samples to the amount measured in the current sample. For example, assume samples 1, 2 and 3 (500 µl each) were removed sequentially from the same plate (10 ml total volume), and had assay values 10, 20 and 30 pg/.1 ml, respectively. Adjusted value for sample 2 would be 20 pg + .5 pg (500 µl removed from 10 ml corresponds to 5% of the concentration on sample 1), which equals 20.5 pg. Similarly, adjusted value for sample 3 should be 30 + .5 + 1 pg (5% concentration sample 1 + 5% concentration unadjusted sample 2) totaling 31.5 pg. Further adjustment on concentrations of $PGF_{2\alpha}$ was performed to account for potential differences in cell number due to treatment effects. Total cell protein was determined as described below, and adjusted total $PGF_{2\alpha}$ values per well were expressed on a per µg of protein basis. Final values of $PGF_{2\alpha}$ production per well were adjusted for previous removal and expressed on a protein basis (total $PGF_{2\alpha}$, pg/µg protein), were used for statistical analyses (see below).

Preparation of Extracts

At the end of cultures, plates were transported to a cold room (4 C) and cells were washed twice in ice cold PBS. Whole cell extracts (WCE) were obtained as described in Chapter 5. Protein concentrations were determined by the Bradford method, as described in Chapter 4, in WCE diluted 1:50 (20 µl sample in 980 µl PBS).

Immunoblotting

Volumes of WCE from each plate corresponding to 20 µg of protein were loaded onto duplicate 7.5% acrylamide gels, submitted to SDS-PAGE, and electrophoretic transfer of proteins to nitrocellulose membranes as described in Chapter 5. Membranes were separately probed with antibodies against either COX-2 [1:500 dilution in Tris-buffered saline (TBS)] and PLA_2 (1:250 dilution in TBS). Secondary antibody was anti-rabbit IgG (1:5000 dilution in TBS containing .1% tween 20). Proteins were detected by ECL and analyzed by densitometry. To validate specificity of antibodies for PLA_2 and COX-2, samples used in Experiment 3 (positive for PLA_2 and COX-2; see below) were submitted to SDS-PAGE and transferred to a nitrocellulose membrane. Membrane was blocked with 5% non-fat dried milk in TBS and incubated with 8 µg of -rabbit IgG (equivalent amount of IgG present in 1:250 dilution of anti-PLA_2 antibody; diluted

in 5% non-fat dried milk in TBS) for 2 hours. Remaining immunoblotting and ECL procedures were conducted as described above.

Experimental Designs

Experiment 1. Sixteen 100 mm plates were assigned randomly to receive bIFN-τ (0 or 50 ng/ml) or PDBu (0 or 100 ng/ml) in quadruplicate, in a two-by-two factorial design, for 24 hours. Medium was sampled (500 μl) immediately before addition of treatments (0-h sample), after 12 and 24 hours of treatment. Samples were stored at -20 C for $PGF_{2\alpha}$ analysis. After the 24 h-sample, remaining medium was discarded and cells were harvested for extraction of proteins for immunoblots (WCE; two plates per treatment).

Experiment 2. Ten 100 mm plates were assigned randomly to receive medium alone (control, 3 plates), PDBu (100 ng/ml, 4 plates) or PDBu in combination with bIFN-τ (50 ng/ml, 4 plates) for 12 hours. Medium was sampled (500 μl) immediately before addition of treatments (0-h sample), after 3, 6, 9 and 12 hours of treatment and stored at -20 C for $PGF_{2\alpha}$ analysis. Following each sample collection time, cells from each treatment were photographed digitally using the imaging apparatus described on Chapter 4. After collection of 12 h-samples, remaining medium was discarded and cells were harvested for extraction of proteins for immunoblots with antiserum to PLA_2 and COX-2 for all plates.

Experiment 3. Twenty seven 100 mm plates were assigned randomly to receive medium alone (control, 9 plates), PDBu (100 ng/ml, 9 plates) or PDBu in combination with bIFN-τ (50 ng/ml, 9 plates). Medium was sampled (500 μl) after 1, 2, 3, 4, 5 and 6 hours of treatment and stored at -20 C for $PGF_{2\alpha}$ analysis. After collection of 6-hour sample, 3 plates per treatment were used for protein extracts (WCE).

Experiment 4. Twelve wells in two 6-well plates were assigned in triplicate to receive medium alone (control), PDBu (100 ng/ml), PDBu in combination with bIFN-τ (50 ng/ml) or PDBu for 3 hours and PDBu in combination with bIFN-τ for the next 3 hours. Medium was sampled (250 μl) immediately before addition of treatments (0-h sample), after 1, 2, 3, 4, 5 and 6 hours of treatment. Medium was stored at -20 C for $PGF_{2\alpha}$ analyses. In the last treatment (PDBu+bIFN-τ-3h), 150 ng of bIFN-τ (amount required for a 50 ng/ml final concentration in the well that contains 3 ml of medium) was added to wells mixed in 250 μl of medium (also containing 100 ng/ml PDBu) to replace medium removed for the 3-hour sample. $PGF_{2\alpha}$ concentrations were not adjusted for well protein content in this experiment.

Statistical Analysis

Data were analyzed by least squares analysis of variance, using the procedure GLM from SAS (SAS, 1988). Each experiment was analyzed

individually. For $PGF_{2\alpha}$, data were analyzed as a split-plot design. Independent variables in the mathematical models were: treatment, dish (or well) within treatment, time, treatment by time and residual error. Effects of treatment were tested using dish within treatment as the error term. Heterogeneity of variance among treatments was tested by the maximum F-ratio test (f max; Hartley, 1950) and found significant in all experiments. Since this invalidates assumptions for analysis of variance, data were transformed to $\log_{10}$ and re-tested for f max. Transformations effectively eliminated heterogeneity of variance for Experiments 2, 3 and 4, but not for Experiment 1. On that experiment, the Box-Cox procedure for data transformation was applied (Peltier et al., 1998) and a λ value of 0.4 was found to eliminate heterogeneity of variance. Data were analyzed after appropriate transformations. However, for the sake of clarity, data are presented as untransformed values. Means were compared as a series of pre-planned orthogonal contrasts.

In Experiment 1, only 12 and 24 hour data were used for analysis. Due its factorial nature, contrasts were bIFN-τ and bIFN-τ+PDBu vs. control and PDBu (tests the effects of bIFN-τ), PDBu and bIFN-τ+PDBu vs. control and bIFN-τ (tests the effects of PDBu) and, control and bIFN-τ+PDBu vs. bIFN-τ and PDBu (tests the interaction of PDBu and bIFN-τ effects). Contrasts were calculated using dish within treatment as the error term. Treatment by time contrasts were also calculated.

In Experiment 2 treatment contrasts were (c1) control vs PDBu and bIFN-τ + PDBu and (c2) PDBu vs. bIFN-τ + PDBu. Contrasts c1 and c2 were calculated using dish within treatment as the error term. Time contrasts were 0 vs. 3, 6, 9, and 12 (c3), 3 vs. 6, 9 and 12 (c4), 6 vs. 9 and 12 (c5) and 9 vs. 12 (c6). Treatment by time interactions were obtained by multiplying c1 and c3, c1 and c4, c1 and c5, c1 and c6, c2 and c3, c2 and c4, c2 and c5, c2 and c6.

In Experiment 3, treatment contrasts were the same as for Experiment 2 but time contrasts were 1 vs. 2, 3, 4, 5 and 6 (c7), 2 vs, 3, 4, 5 and 6 (c8), 3 vs. 4, 5 and 6 (c9), 4 vs 5 and 6 (c10), 5 vs. 6 (c11). Treatment by time interactions were obtained by sequentially multiplying c1 and c2 by c7, c8, c9, c10 and c11, as exemplified for Experiment 2.

In Experiment 4, treatment contrasts were control vs PDBu, PDBu + bIFN-τ and PDBu + bIFN-τ - 3h (c12), PDBu vs PDBu + bIFN-τ and PDBu + bIFN-τ -3h (c13), PDBu + bIFN-τ vs. PDBu + bIFN-τ-3h (c14). Contrasts c12, c13 and c14 were calculated using dish within treatment as the error term. Time contrasts were 0 vs 1, 2, 3, 4, 5 and 6 (c15), c7, c8, c9, c10 and c11. Treatment by time interactions were obtained by sequentially multiplying c12, c13 and c14 by c15, c7, c8, c9, c10 and c11, as exemplified for Experiment 2. In Experiment 4, a separate analysis was performed in which only PDBu and PDBu + bIFN-τ-3h treatments were included. This analysis was conducted on untransformed data, since significant heterogeneity of variance was not detected. The $PGF_{2\alpha}$ secretion data were further analyzed by homogeneity of regression. This

approach allowed us to (1) estimate order of regression of curves for each treatment within experiments, so we could identify changes in secretion rate overtime, (2) determine regression equations for each treatment within experiments, and (3) perform orthogonal comparison of $PGF_{2\alpha}$ secretion among treatments within experiments.

For abundance of COX-2 and PLA_2, mathematical model included only the effect of treatment. Orthogonal contrasts used for mean comparisons were the same used for $PGF_{2\alpha}$ in Experiment 1, and c1 and c2 for Experiments 2 and 3, respectively.

Results

Experiment 1

Secretion of $PGF_{2\alpha}$. Secretion of $PGF_{2\alpha}$ at time 0 was negligible (<0.3 pg/μg protein) for all treatments (Figure 7-1). Long term treatment of BEND cells for 12 or 24 hours with PDBu stimulated a pronounced release of $PGF_{2\alpha}$ (effect of PDBu; $p<0.01$). In contrast, bIFN-τ reduced secretion of $PGF_{2\alpha}$ (effect of bIFN-τ; $p<0.01$). Furthermore, bIFN-τ attenuated the PDBu-induced release of $PGF_{2\alpha}$ both at 12 and 24 hours of treatment (interaction between PDBu and bIFN-τ, $p<0.01$). Moreover, this attenuation was less at 24 hours compared to 12 hours (treatment by time interaction; $p<0.01$).

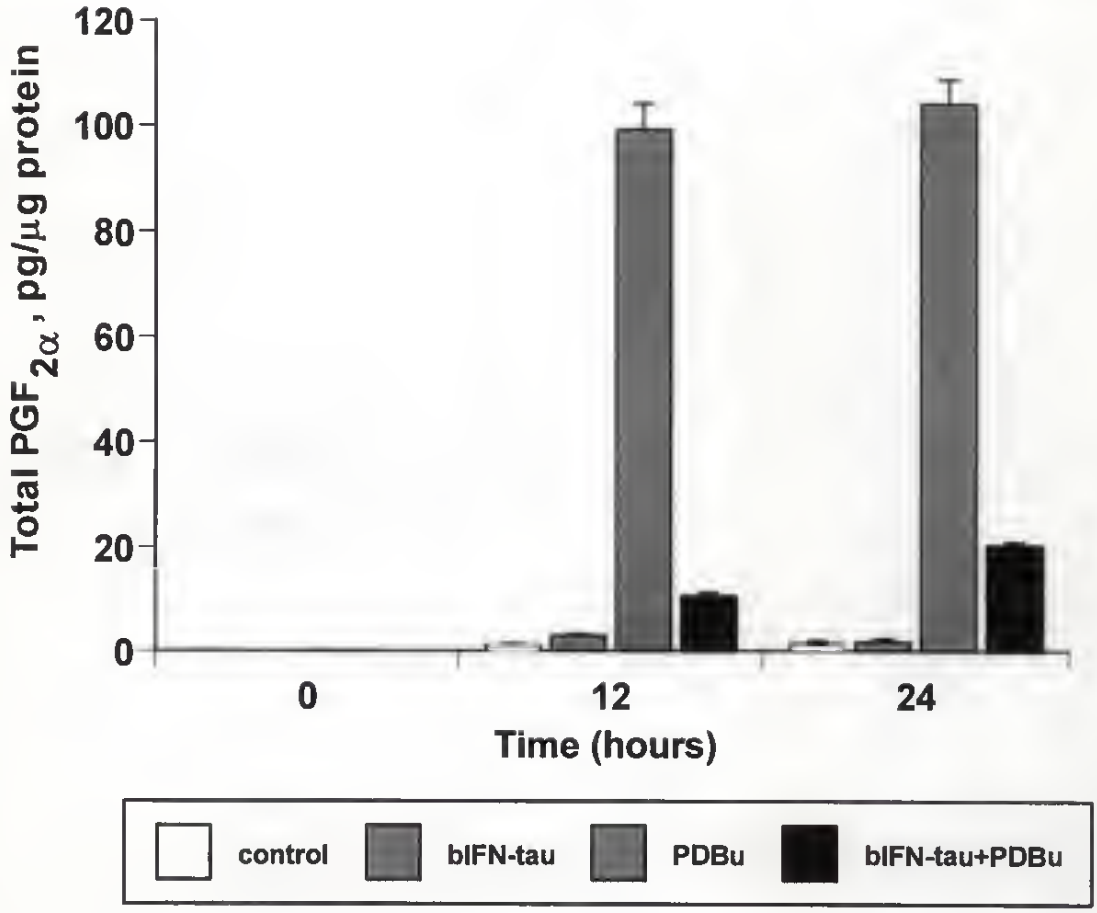


Figure 7-1. Experiment 1. Least squares means and SE of total $PGF_{2\alpha}$ in medium conditioned by BEND cells treated with medium alone (control), bIFN-τ (50 ng/ml; bIFN-tau), phorbol 12,13 dibutyrate (100ng/ml; PDBu) or bIFN-τ and PDBu. Samples were removed before treatments were added (0 hours), 12 and 24 hours after.

COX-2 and PLA_2 immunoblotting. Incubation of membranes with rabbit IgG yielded a clear ECL exposure, suggesting that non-specific binding was negligible in the immunoblotting procedures used (data not-shown). There were low abundances of both COX-2 (Figure 7-2) and PLA_2 (Figure 7-3) on cells incubated with medium alone or with bIFN-τ, but treatment with PDBu stimulated synthesis of both proteins. Incubations with bIFN-τ in combination with PDBu

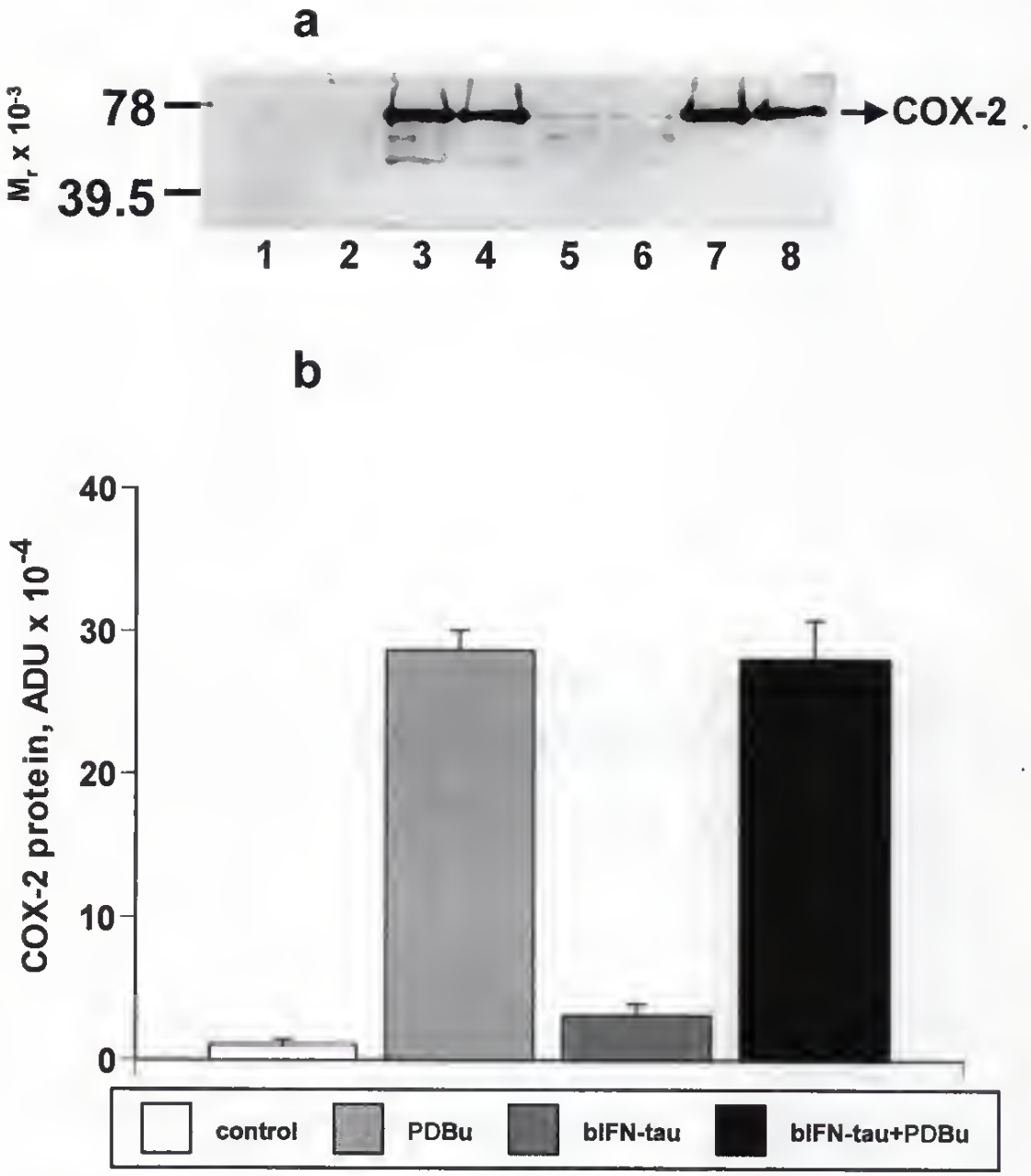


Figure 7-2. Experiment 1. Immunoblotting analysis of **COX-2** in whole cell extracts from BEND cells treated with medium alone (control), bIFN-τ (50 ng/ml; bIFN-tau), phorbol 12,13 dibutyrate (100ng/ml; PDBu) or bIFN-τ and PDBu for 24 hours.

a) Enhanced chemiluminescence (ECL) exposure of abundance of **COX-2** (control: lanes 1 and 2; PDBu: lanes 3 and 4; bIFN-τ lanes 5 and 6; bIFN-τ and PDBu: lanes 7 and 8); b) Least squares means and SE of abundance of **COX-2** arbitrary densitometric units (ADU).

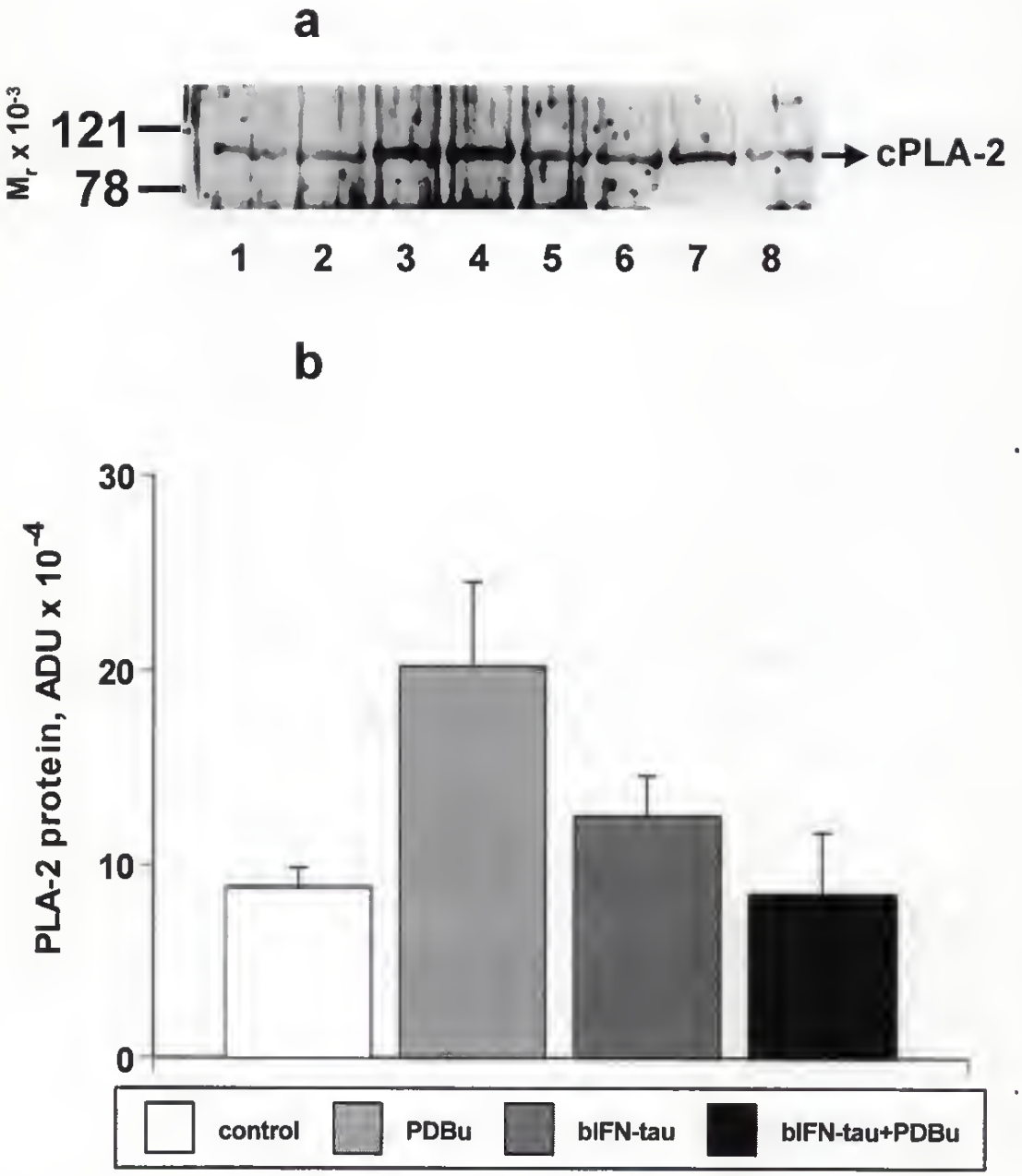


Figure 7-3. Experiment 1. Immunoblotting analysis of **PLA-2** in whole cell extracts from BEND cells treated with medium alone (control), bIFN-τ (50 ng/ml; bIFN-tau), phorbol 12,13 dibutyrate (100ng/ml; PDBu) or bIFN-τ and PDBu for 24 hours.

a) Enhanced chemiluminescence (ECL) exposure of abundance of **PLA-2** (control: lanes 1 and 2; PDBu: lanes 3 and 4; bIFN-τ lanes 5 and 6; bIFN-τ and PDBu: lanes 7 and 8); b) Least squares means and SE of abundance of **PLA-2** arbitrary densitometric units (ADU).

decreased abundance of PLA_2 compared to PDBu alone. In contrast, there was no effect of bIFN-τ on the PDBu-induced COX-2 protein expression.

Experiment 2

Secretion of $PGF_{2\alpha}$. Similar to Experiment 1, treatment with PDBu stimulated an acute release of $PGF_{2\alpha}$ compared to controls (Figure 7-4). The PDBu-induced $PGF_{2\alpha}$ secretion was noticed as early as 3 hours and increased at a fast rate between 0 and 6 hours (47.4 pg/ μg protein/6 hours). Further increase in $PGF_{2\alpha}$ release was noticed, but occurred at a lower rate (14.7 pg/ μg of protein/6 hours). Bovine IFN-τ attenuated the PDBu-induced release of $PGF_{2\alpha}$ at 3, 6, 9 and 12 hours of treatment. Significant time by treatment contrasts [c2 x c3, (p<0.01); c2 x c5 (p<0.04); c2 x c6 (p<0.01)] indicate that bIFN-τ decreased the rate of increase on PDBu-stimulated $PGF_{2\alpha}$ secretion. Furthermore, analysis of homogeneity of regression indicated that $PGF_{2\alpha}$ secretion could be represented by third order curves (R^2: .968), which confirmed changes in secretion rates during the experiment. Equations for each treatment were: $Y=-0.5808+7.3175 \times X+0.21227 \times X^2-0.0325 \times X^3$ (PDBu); $Y=-0.1259 \times X-0.1961 \times X^2+0.011 \times X^3$ (PDBu + bIFN-τ); where Y represents secretion of $PGF_{2\alpha}$, and X is a given time point in the experiment. Orthogonal comparisons of curves confirmed that PDBu stimulated $PGF_{2\alpha}$ secretion, and that bIFN-τ attenuated that effect (p<0.01).

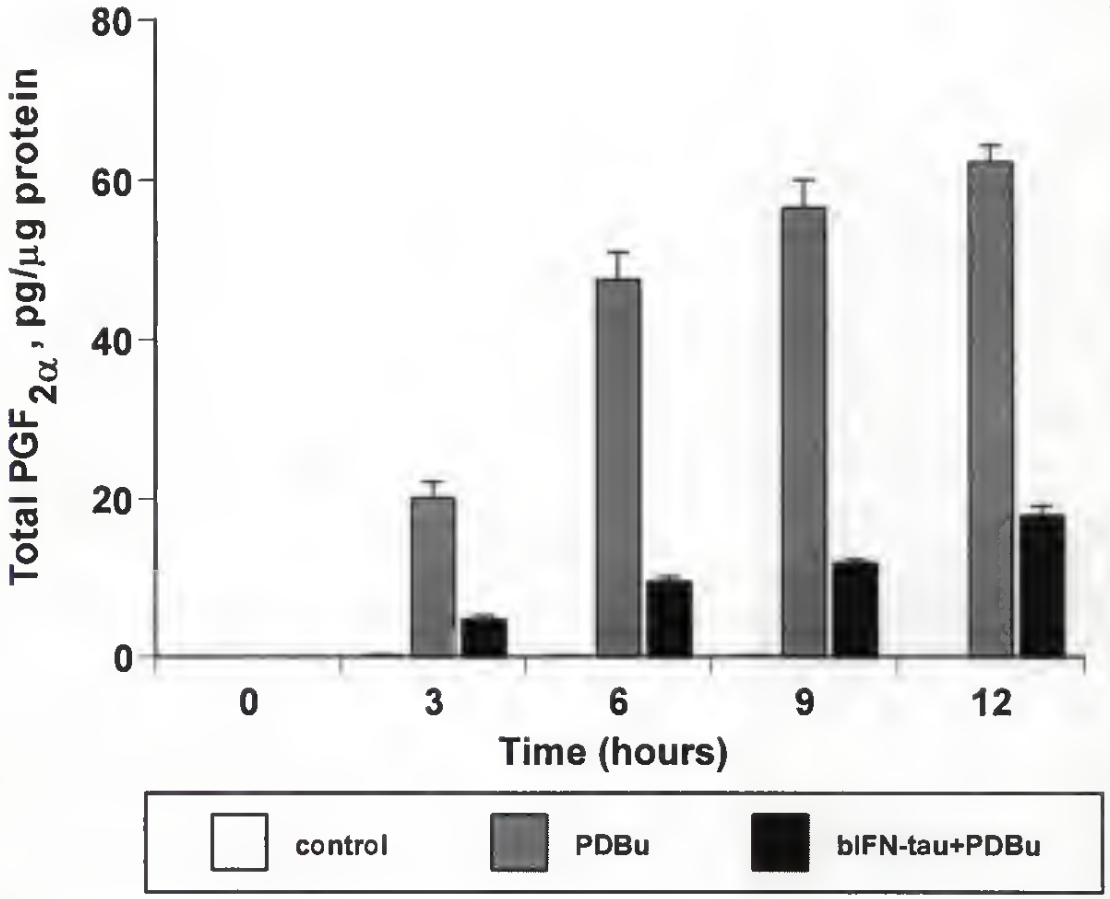


Figure 7-4. Experiment 2. Least squares means and SE of concentrations of $PGF_{2\alpha}$ in medium conditioned by BEND cells treated with medium alone (control), phorbol 12,13 dibutyrate (100ng/ml; PDBu) or bIFN-τ (50 ng/ml; bIFN-tau) and PDBu. Samples were removed before treatments were added (0 hours), 3, 6, 9 and12 hours after.

COX-2 and PLA_2 immunoblotting. Similar to Experiment 1, there were very low abundances of both COX-2 ($p<0.01$; Figure 7-5) and PLA_2 ($p<0.04$; Figure 7-6) in cells incubated with medium alone. However, treatment with PDBu stimulated synthesis of both proteins. In comparison with Experiment 1, treatment with bIFN-τ in combination with PDBu decreased abundance of both COX-2 ($p<0.01$) and PLA_2 ($p<0.05$) compared to PDBu alone at 12 hours.

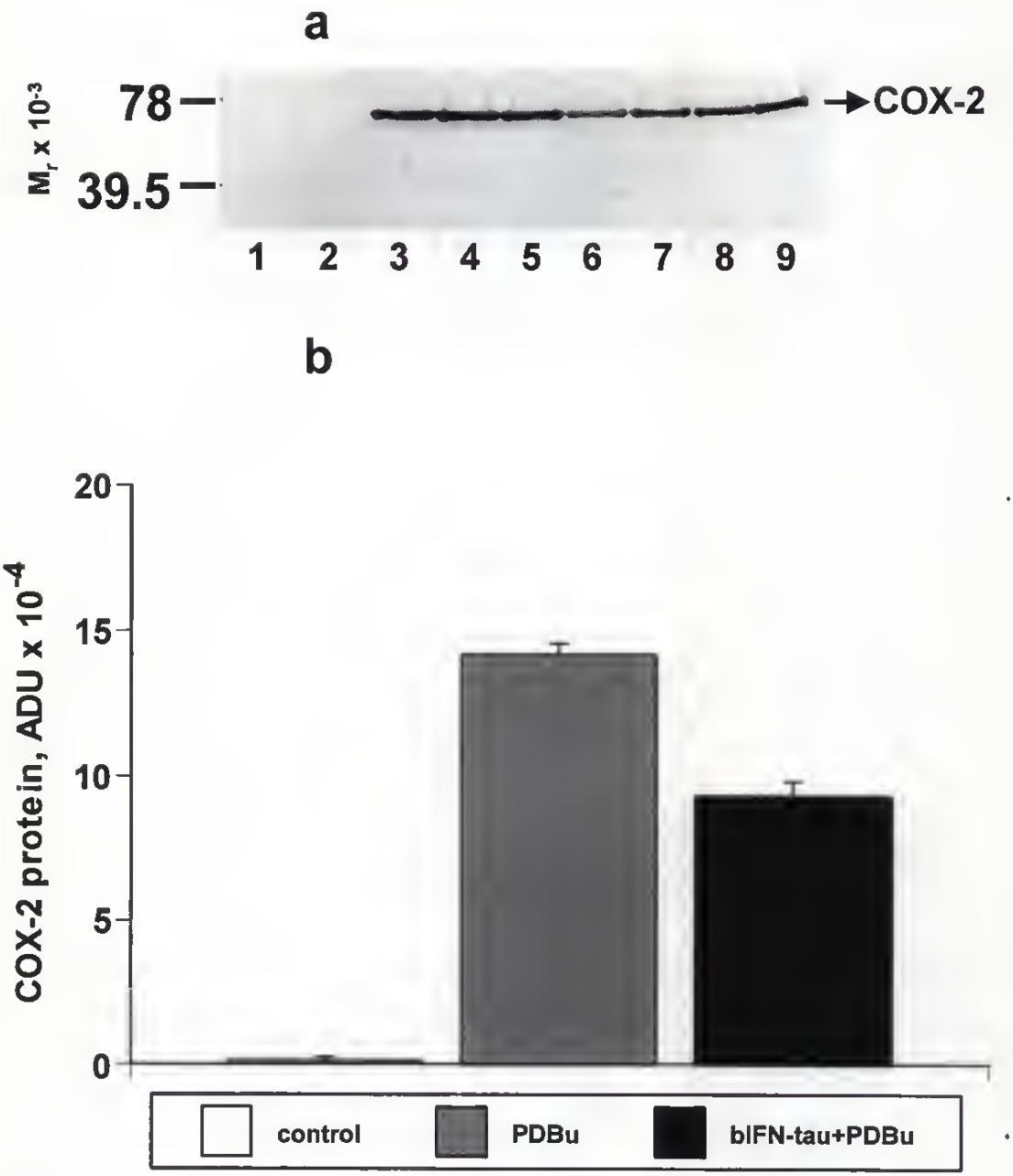


Figure 7-5. Experiment 2. Immunoblotting analysis of **COX-2** in whole cell extracts from BEND cells treated with medium alone (control), phorbol 12,13 dibutyrate (100ng/ml; PDBu) or bIFN-τ (50 ng/ml; bIFN-tau) and PDBu for 12 hours.

a) Representative enhanced chemiluminescence (ECL) exposure of abundance of **COX-2** (control: lanes 1 and 2; PDBu: lanes 3, 4 and 5; bIFN-τ and PDBu: lanes 6, 7, 8 and 9); b) Least squares means and SE of abundance of **COX-2** arbitrary densitometric units (ADU).

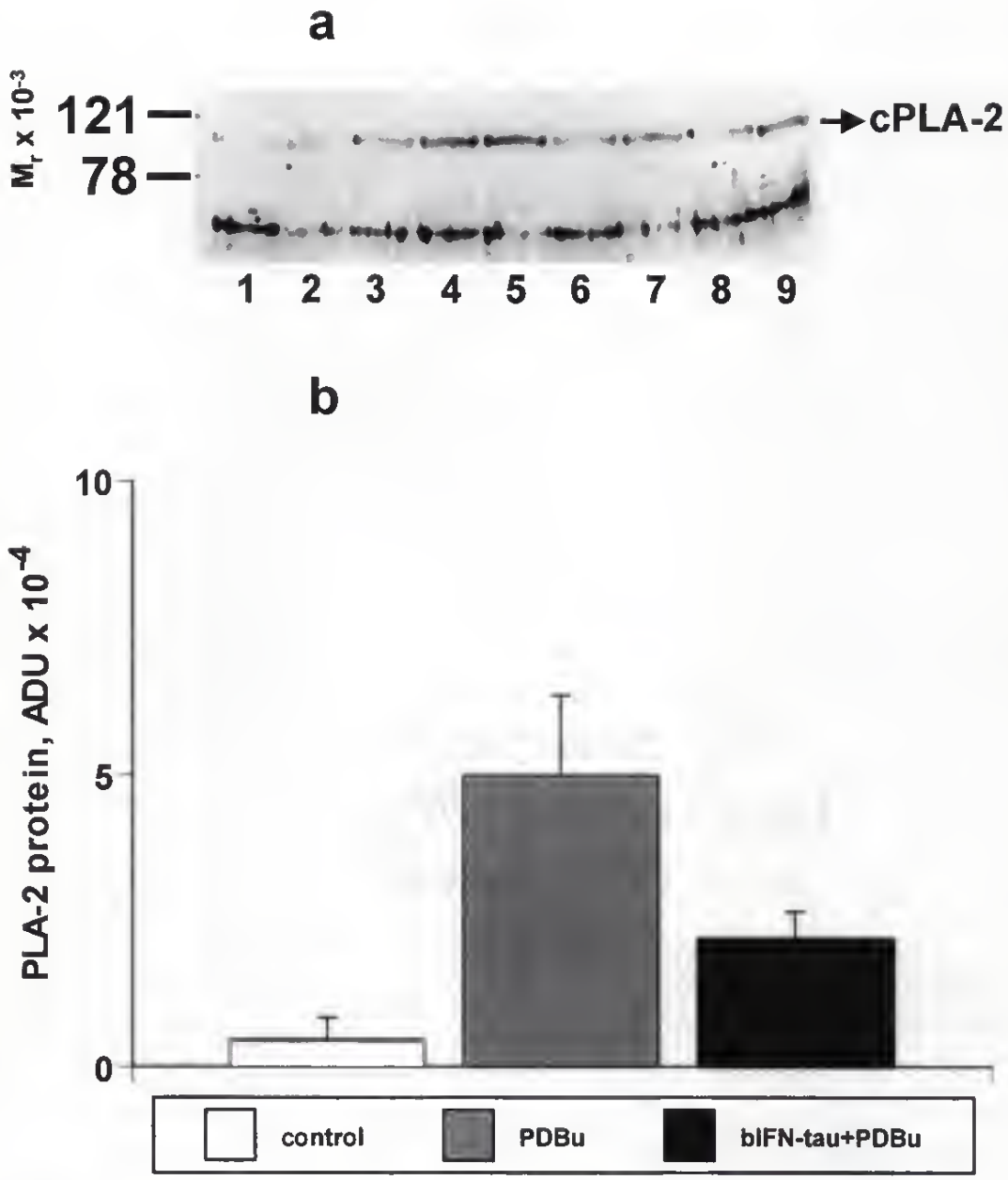


Figure 7-6. Experiment 2. Immunoblotting analysis of **PLA-2** in whole cell extracts from BEND cells treated with medium alone (control), phorbol 12,13 dibutyrate (100ng/ml; PDBu) or bIFN-τ (50 ng/ml; bIFN-tau) and PDBu for 12 hours.

a) Representative enhanced chemiluminescence (ECL) exposure of abundance of **PLA-2** (control: lanes 1 and 2; PDBu: lanes 3, 4 and 5; bIFN-τ and PDBu: lanes 6, 7, 8 and 9); b) Least squares means and SE of abundance of **PLA-2** arbitrary densitometric units (ADU).

Cell morphology. The PDBu both alone and in combination with bIFN-τ induced clear changes in cell morphology overtime (Figure 7-7). Cells became clustered and were connected by arm-like projections that were evident by 3 hours. In contrast, cell morphology did not change in controls. Despite such changes, total protein content was similar among treatments (835.4, 936.2 and 787.6±52.3 for control, PDBu and PDBu + bIFN-τ respectively).

Experiment 3

Secretion of $PGF_{2\alpha}$. This experiment characterized the short term rise in $PGF_{2\alpha}$ secretion in response to PDBu stimulation, in presence and absence of bIFN-τ (Figure 7-8). Noticeable stimulation by PDBu was detected after 2 hours of exposure. There was a steady and faster rate increase in PDBu-induced secretion of $PGF_{2\alpha}$ throughout the experiment compared to PDBu + bIFN-τ. Significant treatment by time contrasts (c1 x c7, c1 vs c7, c1 x c8, c1 x c9 and c1 x c10; $p<.01$) indicates that basal accumulation of $PGF_{2\alpha}$ was negligible throughout the experiment. In contrast, other treatment by time contrasts (c2 x c9, c2 x c10, c2 x c11) were not significant ($p>.1$), indicating that trends in increase of PDBu-stimulated $PGF_{2\alpha}$ were similar in presence or absence of bIFN-τ. Analysis of homogeneity of regression indicated that $PGF_{2\alpha}$ secretion could be represented by fourth order curves (R^2: .982), which confirmed changes in secretion rates during the experiment. Equations for each treatment were: $Y=-2.51+7.66 \times X-4.2 \times X^2+0.91 \times X^3-0.07 \times X^4$ (control); $Y=23.59-43.3 \times X+27.63 \times$

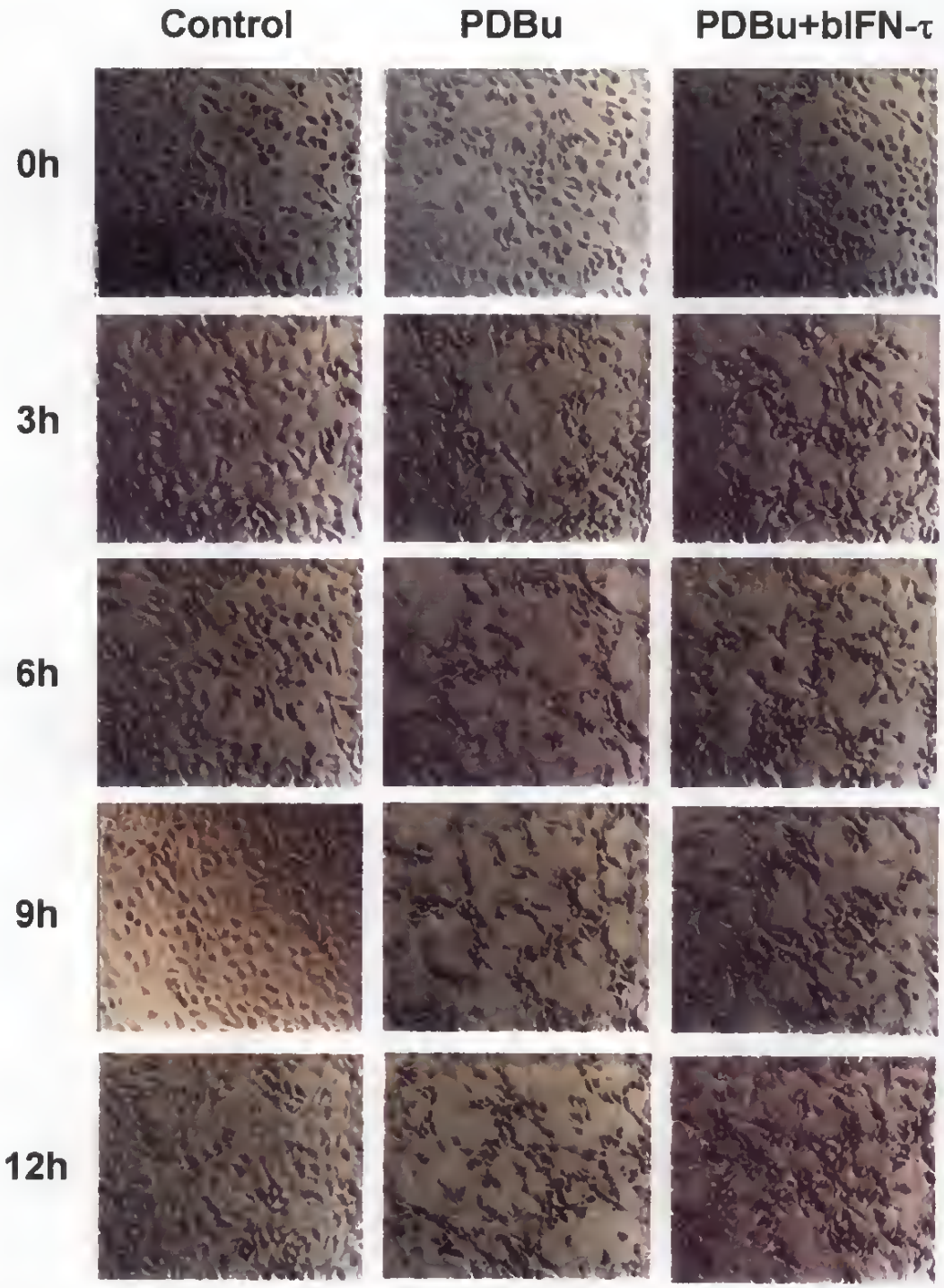


Figure 7-7. Experiment 2. Changes in morphology over time (vertical axis, hours) of BEND cells treated with medium alone (control), phorbol 12,13 dibutyrate (PDBu; 100ng/ml) or bIFN-τ (50 ng/ml) and PDBu (horizontal axis). Magnification: 100x.

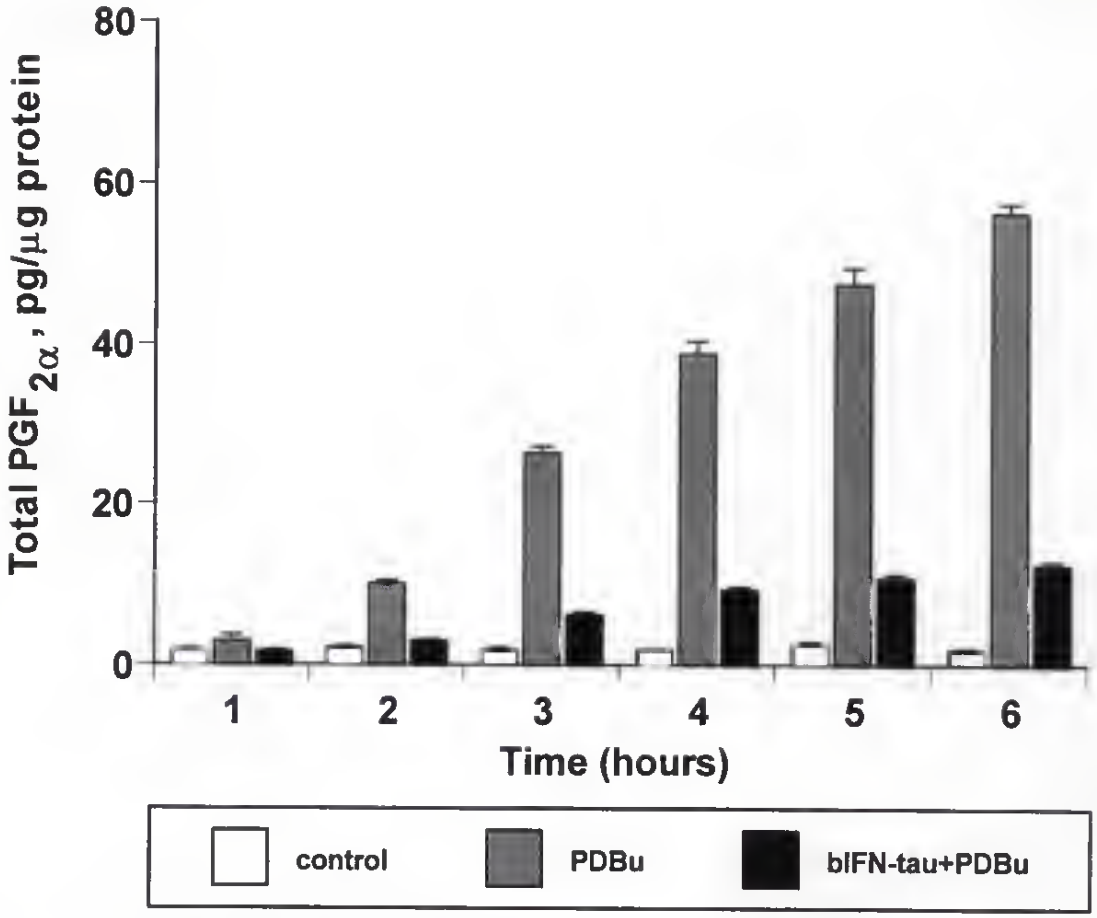


Figure 7-8. Experiment 3. Least squares means and SE of concentrations of $PGF_{2\alpha}$ in medium conditioned by BEND cells treated with medium alone (control), phorbol 12,13 dibutyrate (100ng/ml; PDBu) or bIFN-τ (50 ng/ml; bIFN-tau) and PDBu. Samples were removed before treatments were added (0 hours), 1, 2, 3, 4, 5 and 6 hours after.

X^2-5.4 x X^3+0.35 x X^4 (PDBu); Y=6.88-10.46 x X+6.34 x X^2-1.21 x X^3+0.08 x X^4 (PDBu + bIFN-τ); where Y represents secretion of $PGF_{2\alpha}$, and X is a given time point in the experiment. Orthogonal comparisons of curves confirmed that PDBu stimulated $PGF_{2\alpha}$ secretion, and that bIFN-τ attenuated that effect (p<0.01).

COX-2 and PLA_2 immunoblotting. In agreement with previous experiments, there was only a low, basal level of COX-2 (Figure 7-9) and PLA_2

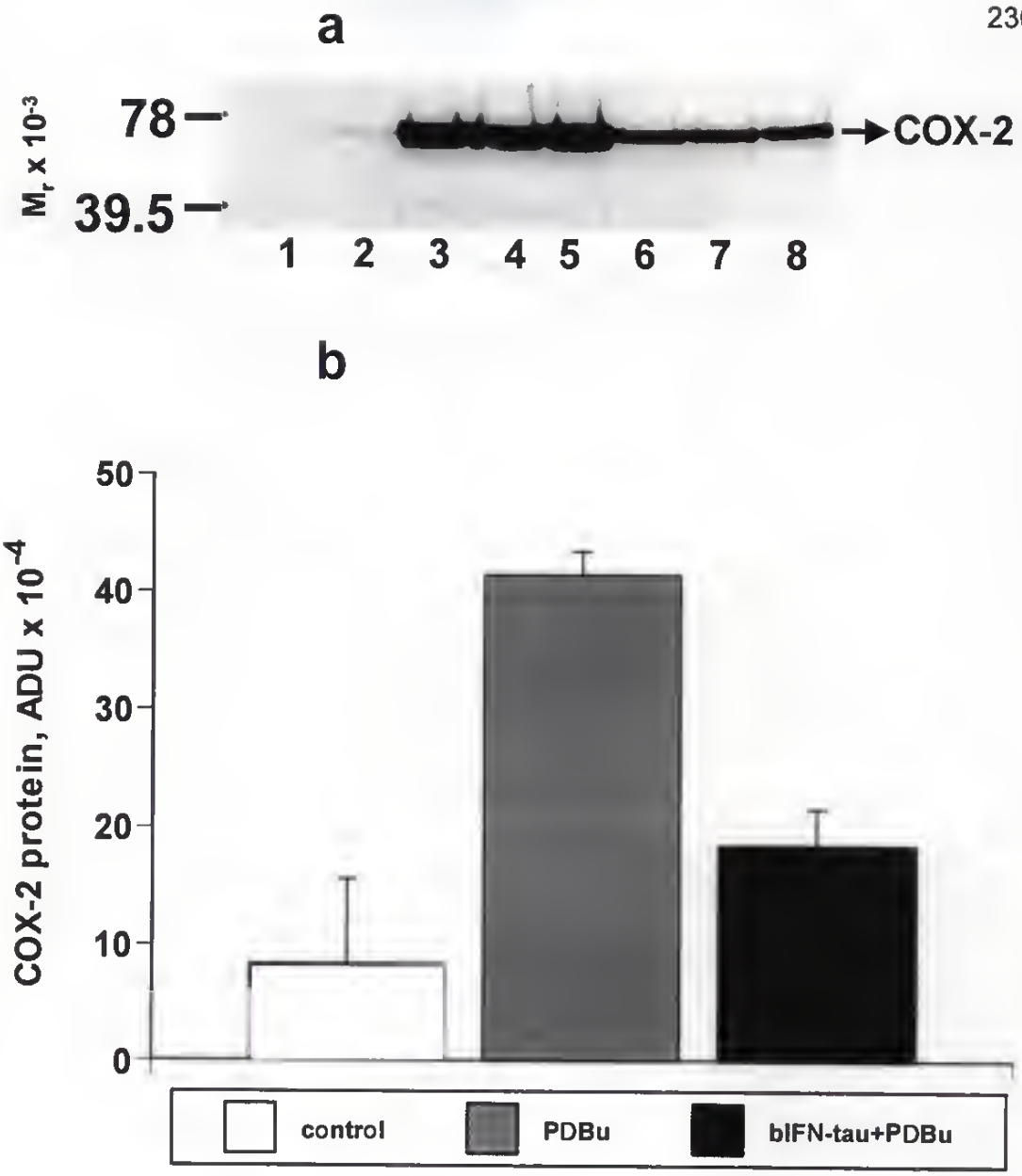


Figure 7-9. Experiment 3. Immunoblotting analysis of **COX-2** in whole cell extracts from BEND cells treated with medium alone (control), phorbol 12,13 dibutyrate (100ng/ml; PDBu) or bIFN-τ (50 ng/ml; bIFN-tau) and PDBu for 6 hours.

a) Representative enhanced chemiluminescence (ECL) exposure of abundance of **COX-2** (control: lanes 1 and 2; PDBu: lanes 3, 4 and 5; bIFN-τ and PDBu: lanes 6, 7 and 8); b) Least squares means and SE of abundance of **COX-2** arbitrary densitometric units (ADU).

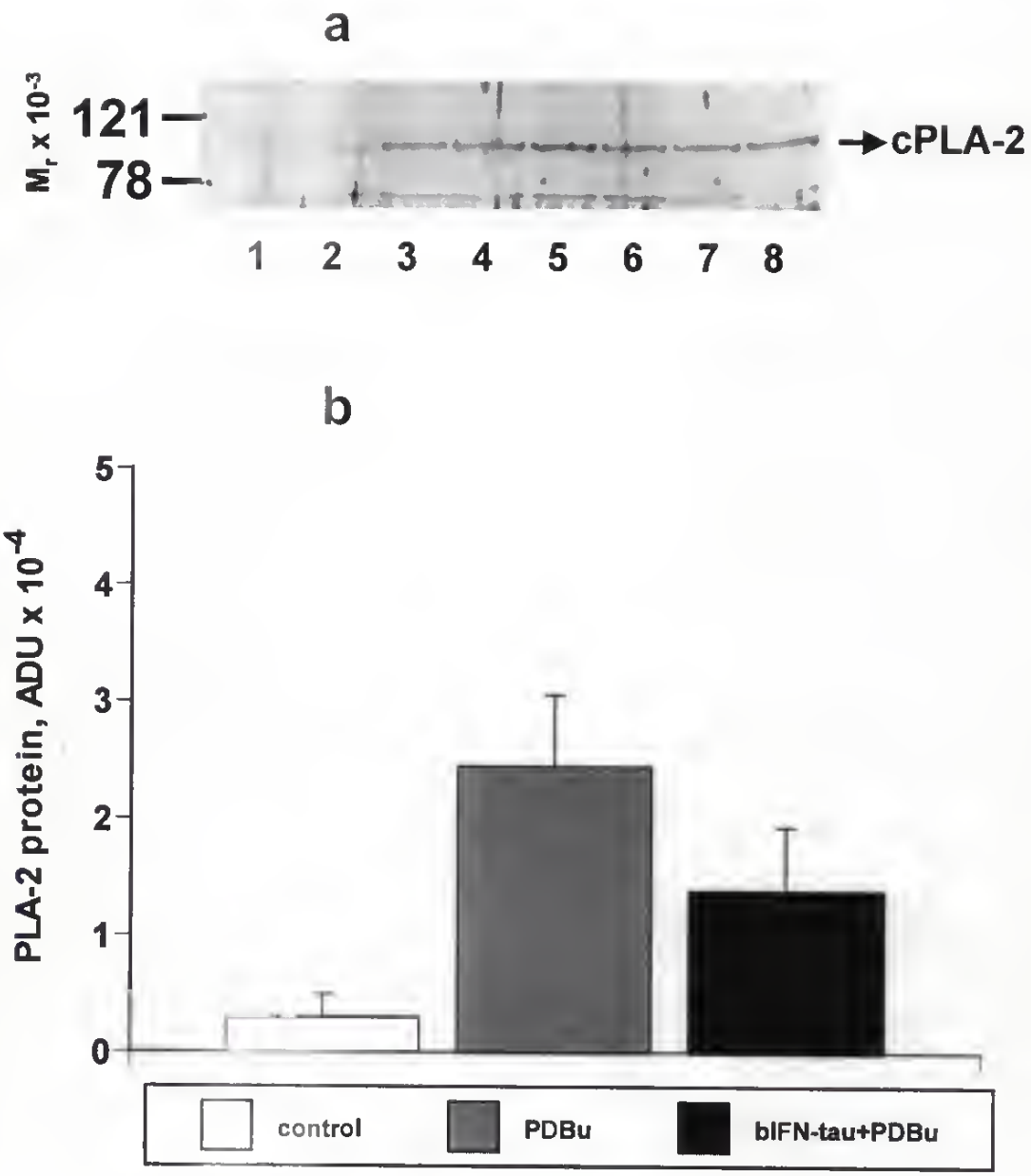


Figure 7-10. Immunoblotting analysis of **PLA-2** in whole cell extracts from BEND cells treated with medium alone (control), phorbol 12,13 dibutyrate (100ng/ml; PDBu) or bIFN-τ (50 ng/ml; bIFN-tau) and PDBu for 6 hours.

a) Representative enhanced chemiluminescence (ECL) exposure of abundance of **PLA-2** (control: lanes 1 and 2; PDBu: lanes 3, 4 and 5; bIFN-τ and PDBu: lanes 6, 7 and 8); b) Least squares means and SE of abundance of **PLA-2** arbitrary densitometric units (ADU).

(Figure 7-10) in cells incubated with medium alone. Presence of PDBu treatment stimulated synthesis of both proteins ($p<.01$ and $p<.08$; contrast c1 for COX-2 and PLA_2 respectively at 6 hours). However, adding bIFN-τ markedly reduced PDBu-induced expression of COX-2 ($p<.01$; contrast c2) and a numerical but non-significant decrease in PLA_2 ($p>.2$; contrast c2).

Experiment 4

Secretion of $PGF_{2\alpha}$. Patterns of $PGF_{2\alpha}$ secretion into culture medium over time were similar to ones described in Experiment 3 for controls, PDBu and PDBu + bIFN-τ treatments (Figure 7-11). Treatment with PDBu stimulated $PGF_{2\alpha}$ secretion through 6 hours and this response was attenuated markedly with the concurrent addition of bIFN-τ. Addition of bIFN-τ after a 3-hour exposure to PDBu caused a decrease in PDBu-stimulated $PGF_{2\alpha}$ secretion, which is noticeable at the 5-hour sample and becomes even more pronounced at the 6-hour sample. Moreover, rate of accumulation of $PGF_{2\alpha}$ in medium between 4 and 6 hour is greater in absence of bIFN-τ is (1201 pg/ml/2 hours for PDBu vs. 442 pg/ml/2 hours for PDBu + bIFN-τ-3h). Furthermore, when PDBu and PDBu + bIFN-τ-3h treatments were analyzed without other treatments, there was a significant time by treatment interaction ($p<0.01$), indicating that bIFN-τ effectively suppressed PDBu-stimulated $PGF_{2\alpha}$ secretion, even when added after PDBu treatment had been initiated. Analysis of homogeneity of regression

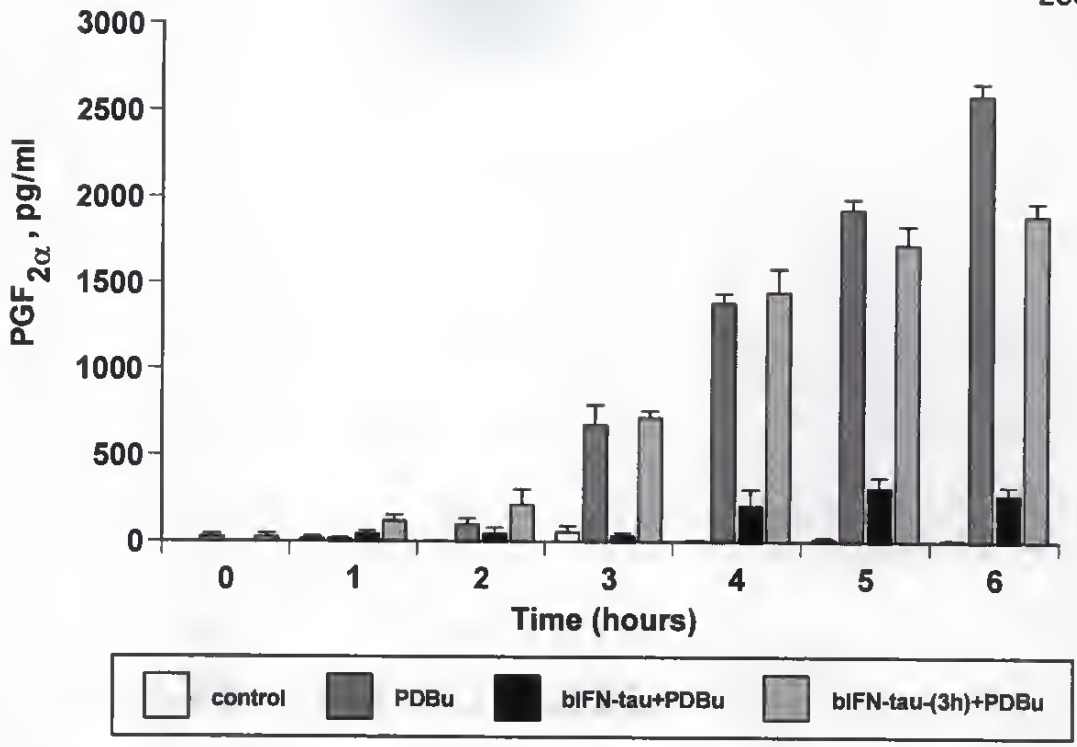


Figure 7-11. Experiment 4. Least squares means and SE of concentrations of $PGF_{2\alpha}$ in medium conditioned by BEND cells treated with medium alone (control), phorbol 12,13 dibutyrate (100ng/ml; PDBu),bIFN-τ (50 ng/ml; bIFN-tau) and PDBu and bIFN-τ added 3 hours after other treatments started and PDBu (see text). Samples were removed before treatments were added (0 hours), 1, 2, 3, 4, 5 and 6 hours after.

indicated that $PGF_{2\alpha}$ secretion could be represented by third order curves (R^2: .954), which confirmed changes in secretion rates during the experiment. Equations for each treatment were: $Y=-11.96+29.28 \times X-6.89 \times X^2+0.41 \times X^3$ (control); $Y=46.55-319.2 \times X+229.1 \times X^2-17.64 \times X^3$ (PDBu); $Y=22.86-67.02 \times X+45.2 \times X^2-4.42 \times X^3$ (PDBu + bIFN-τ); $Y=58.27-287.4 \times X+260.05 \times X^2-26.92 \times X^3$ (PDBu + bIFN-τ-3 hour); where Y represents secretion of $PGF_{2\alpha}$, and X is a given time point in the experiment. Orthogonal comparisons of curves confirmed that PDBu stimulated $PGF_{2\alpha}$ secretion, and that bIFN-τ attenuated that effect

($p<0.01$). Moreover, curves representing secretion of $PGF_{2\alpha}$ induced by PDBu and PDBu + bIFN-τ were different ($p<0.01$).

Discussion

The present chapter describes a cell culture-based model that is adequate for studying effects of bIFN-τ on $PGF_{2\alpha}$ secretion in the endometrium. This system has the basic components for such studies, which are (1) responsiveness to a stimulator of $PGF_{2\alpha}$ synthesis (i.e., PDBu) and (2), responsiveness to bIFN-τ, which blocks a stimulation in $PGF_{2\alpha}$. Using this system, it was determined that a bIFN-τ-induced suppression of $PGF_{2\alpha}$ is probably mediated through a decrease in protein expression and or activity of two critical enzymes for synthesis of $PGF_{2\alpha}$: COX-2 and PLA_2. Moreover, bIFN-τ suppression of PDBu-stimulated $PGF_{2\alpha}$ secretion can be achieved quickly (less than 2 hours) and after $PGF_{2\alpha}$ synthesis has been stimulated with PDBu.

BEND cells provide an adequate model for studying regulation of $PGF_{2\alpha}$ in the endometrium. Previous studies utilized endometrial epithelial cells in primary culture harvested from uteri collected from day 15 cyclic cows (Danet-Desnoyers et al., 1994) and day 1 to 4 cyclic cows (Asselin et al., 1997; Asselin et al., 1998; Xiao et al., 1999). Although day 15 cells are preferable in comparison to days 1 to 4 (i.e., cells are from a pre-luteolysis stage of the estrous cycle in which uteri have been primed adequately by progesterone), collection procedures are time

and resource consuming. BEND cells are spontaneously immortalized, and can be used repeatedly for at least 15 passages (see Chapter 5; unpublished author's observations). Moreover, BEND cells originated from day 14 cyclic cows, which provide an adequate model to study mechanisms associated with luteolysis and maternal recognition of pregnancy associated with CL maintenance. Secretion of $PGF_{2\alpha}$ and suppression of such secretion by conceptus-secreted bIFN-τ are hallmark processes during luteolysis and maternal recognition of pregnancy events, respectively. Therefore, an adequate in vitro system to study such events should mimic such processes.

In all experiments reported in the present study, PDBu stimulated $PGF_{2\alpha}$ from BEND cells. Phorbol esters such as PMA have been used previously to study regulation of $PGF_{2\alpha}$ secretion in vitro, and have shown a similar stimulatory effect (Xiao et al., 1999). In the present study, PDBu continuously stimulated $PGF_{2\alpha}$ secretion for up to 12 hours, but further increase between 12 and 24 hours of exposure to PDBu was negligible. Further increases in PDBu-stimulated $PGF_{2\alpha}$ may have occurred due to depletion of arachidonic acid (precursor for $PGF_{2\alpha}$ synthesis), or due to down-regulation of the PDBu stimulatory system. Experiment 2 indicated that maximum $PGF_{2\alpha}$ secretion rate occurs during the first 6 hours. From 6 to 12 hours secretion rate is less. Experiments 3 and 4 suggest that $PGF_{2\alpha}$ is secreted in a constant rate between 3 and 6 hours, and that it takes at least 2 hours for PDBu effects to be detected. This suggests that PDBu activity is, at least initially dependent on de novo protein synthesis, and

not on stimulation of activity of pre-existing enzymes involved in $PGF_{2\alpha}$ production. This agrees with data from Nam and others (1996), which reported that a protein synthesis inhibitor, cyclohexamide, blocked the ability of PDBu to stimulate $PGF_{2\alpha}$ production in astroglial cells. The PDBu had a acute effect on morphology of BEND cells. However, this did not seem to be associated with increased cell mortality, at least based on total protein measurements, which were similar among treatments at the end of cultures.

When added alone to BEND cells, bIFN-τ had negligible effect on $PGF_{2\alpha}$ secretion (Experiment 1). Therefore, this treatment was excluded from subsequent experiments. In contrast, bIFN-τ effectively suppressed the PDBu-induced release of $PGF_{2\alpha}$ in all experiments. However, bIFN-τ was never able to completely suppress PDBu-stimulated $PGF_{2\alpha}$. This could indicate that other conceptus molecules are required to abolish $PGF_{2\alpha}$ completely in vivo. These findings confirm and expand those of Xiao and coworkers (1999). Those authors reported a 4-fold, PMA-induced (2.9 ng/ μg DNA) stimulation of $PGF_{2\alpha}$ secretion compared to controls (0.75 ng/ μg DNA) in a 12-hour experiment. In combination with PMA, bIFN-τ attenuated the $PGF_{2\alpha}$ increase (2 ng/ μg DNA), but only to about 2.7 fold of control values. $PGF_{2\alpha}$ attenuation in the present study was much more dramatic (presence of bIFN-τ reduced PDBu-stimulated $PGF_{2\alpha}$ to one third; Experiment 2, 12 hours).

In summary, the experiments presented provide a useful model for studying bIFN-τ regulation of $PGF_{2\alpha}$ synthesis in the endometrium of cattle. Experiments should be limited to 12-hour duration or less, since no further accumulation of $PGF_{2\alpha}$ was noted for longer time frames. Treatment-induced differences in secretion of $PGF_{2\alpha}$ were only noticed starting 3 hours after administration of PDBu. Therefore, measurements taken before this period may not provide useful information. Although there was no difference in protein content among treatments, it is useful to express $PGF_{2\alpha}$ data relative to some response representative of number of cells present per plate. This cell culture system can be used as a model to answer a number of scientific questions regarding bIFN-τ effects in the endometrium. Such questions include the role of lipid metabolism on bIFN-τ antiluteolytic actions, role of the JAK-STAT pathway and other intracellular signal transduction pathways on bIFN-τ -mediated $PGF_{2\alpha}$ inhibition, bIFN-τ regulation of expression and activity of enzymes involved in the synthesis of $PGF_{2\alpha}$, role of bIFN-τ -induced proteins on inhibition of $PGF_{2\alpha}$ synthesis, potential antiluteolytic effects of conceptus-secreted products other than bIFN-τ etc.

In the present Chapter, cellular mechanisms mediating PDBu-induced and bIFN-τ-suppressed secretion of $PGF_{2\alpha}$ were studied. Specifically, protein expression of PLA_2 and COX-2 were examined over different time frames. Treatment with PDBu strongly stimulated synthesis of both COX-2 and PLA_2

compared to controls, and this was noticed as early as 6 hours (Experiment 3) and lasted for at least 24 hours (Experiment 1). Increased abundance of these enzymes was probably related to their increased cellular activity [abundance (μg of protein) x specific enzymatic activity (activity/μg of protein)], as evaluated through increased $PGF_{2\alpha}$ secretion in medium. Presence of bIFN-τ attenuated PDBu-stimulated PLA_2 and COX-2 protein expression, but such effects were time dependent. Responses of PLA_2 were somewhat variable, but consistently lower than PDBu treatment alone. Although treatment by experiment interactions were not examined (experiments were analyzed independently), visual inspection of data suggests an increase in the ratio of PDBu- to PDBu + bIFN-τ-induced PLA_2 from 6 (Experiment 3) to 24 hours (Experiment 1). Interestingly, the opposite happened for COX-2 abundance. Apparently, bIFN-τ gradually lost its ability inhibit COX-2 expression overtime, and no difference was detected between PDBu and PDBu + bIFN-τ after 24-hour treatments. When related to time trends of $PGF_{2\alpha}$ secretion, these results suggest that differential increases in COX-2 protein expression are associated with increasing $PGF_{2\alpha}$ accumulation overtime as observed for cells treated with the combination of PDBu and bIFN-τ. However, COX-2 levels alone do not explain differences in $PGF_{2\alpha}$ secretion between PDBU and PDBU + bIFN-τ treatments. The proportional decrease in $PGF_{2\alpha}$ is much greater than the proportional decrease in COX-2 abundance for cells treated with PDBu in presence vs. absence of bIFN-

τ. These observations are consistent with those of Xiao and others (1999). They reported that PMA stimulated COX-2 protein expression (about 5 fold higher than control) and that bIFN-τ attenuated that response (to about 2.5 fold higher than control). Moreover, those authors treated cells with oxytocin in presence or absence of bIFN-τ for increasing intervals of time (3 to 24 hours) and measured secretion of $PGF_{2\alpha}$, gene and protein expression of COX-2. Oxytocin stimulated $PGF_{2\alpha}$ accumulation in culture medium throughout the experiment. Message for COX-2 was maximum at 3 hours and gradually decreased overtime, while protein was maximum at 12 hours. Presence of bIFN-τ attenuated all responses measured at all time points. In contrast, Asselin and others (1997) reported no changes in PLA_2 gene expression after treatment of endometrial and stromal cells with bIFN-τ. Moreover, COX-2 expression was upregulated by bIFN-τ. Large doses of bIFN-τ (1 to 20 μg/ml vs 50 ng/ml in the current study) could explain this discrepancy of results. Collectively, it appears that bIFN-τ exerts a complex regulation of both PLA_2 and COX-2 gene expression and activity to modulate $PGF_{2\alpha}$ secretion in BEND cells (see "Hypothetical Model" below).

The fact that inhibitory effects of bIFN-τ on PDBu-induced $PGF_{2\alpha}$ secretion were noted as early as 2 hours (time when first PDBu-induced rise in $PGF_{2\alpha}$ is noted; Experiment 3; Figure 7-8) prompted us to examine how quickly bIFN-τ would be able to suppress synthesis of $PGF_{2\alpha}$ after $PGF_{2\alpha}$ secretion had been

initiated previously by PDBu. We chose to add bIFN-τ after 3 hour-exposure to PDBu because (1) PDBu-induced $PGF_{2\alpha}$ secretion was increasing at a fast rate by this time (Figure 7-8), which indicates that the $PGF_{2\alpha}$ synthesizing machinery was present and functional [also suggested by data from Xiao and coworkers (1999), showing maximum gene expression of COX-2 after 3-hour stimulation with oxytocin) and (2) maximum secretion rate was noticed in the first 6 hours of PDBu stimulation (Experiment 2; Figure 7-4). Decreased rate of secretion of $PGF_{2\alpha}$ 1 hour after addition of bIFN-τ (PDBu + bIFN-τ-3 h treatment, Experiment 4) indicated that bIFN-τ was able to quickly suppress PDBu-induced $PGF_{2\alpha}$. The exact mechanism whereby bIFN-τ exerts its effects is unknown, and are under active investigation at the moment. However it is tempting to speculate that such a quick action is independent of protein synthesis and can occur through novel, previously undescribed cytosolic (i.e., nuclear independent) actions of bIFN-τ.

Next, I will propose a mechanism of bIFN-τ actions in the PDBu-stimulated $PGF_{2\alpha}$ synthesis model. The following discussion makes a distinction between bIFN-τ effects and bIFN-τ modes of action. Effects include the changes in protein expression of PLA_2 and COX-2 examined in the present study. Modes of action include bIFN-τ-stimulated signal transduction, generation of bIFN-τ effector molecules and modification (i.e., activation, deactivation) of pre-existing bIFN-τ-mediator molecules.

Effects of bIFN-τ to inhibit synthesis of $PGF_{2\alpha}$ involving PLA_2 and COX-2 can be explained in terms of attenuation in both gene expression and enzymatic activity (Figure 7-12). Bovine IFN-τ initially inhibited PDBu-induced COX-2 synthesis (Experiment 3) but lost this ability after 24 hours (Experiment 1; Figure 7-2). However, the continued suppression of $PGF_{2\alpha}$ secretion (Figure 7-1) suggests that bIFN-τ must have altered COX-2 enzymatic activity. Two possible mechanisms leading to that effect are activation of bIFN-τ-induced COX-2 intracellular inhibitors and a decrease in the availability of the $PGF_{2\alpha}$ synthesis substrate, arachidonic acid. Regarding the first alternative, Thatcher and coauthors (1994) reported the identification of an endometrial prostaglandin synthesis inhibitor, which acts as a competitive inhibitor of COX-2 activity, as measured by conversion of radiolabeled arachidonic acid into $PGF_{2\alpha}$ in a microsome assay system. This inhibitor has been identified as linoleic acid. Therefore, one could speculate that bIFN-τ increases availability of linoleic acid in BEND cells to inhibit COX-2 activity. Availability of arachidonic acid for $PGF_{2\alpha}$ synthesis is dependent on PLA_2 activity. PLA_2 specifically cleaves arachidonic acid from second position of membrane phospholipids. Bovine IFN-τ continuously suppressed PDBu-stimulated synthesis of PLA_2 in the present study. Although we have no direct measure of PLA_2 enzymatic activity, it is possible that bIFN-τ also suppressed activity of this enzyme as a means to inhibit $PGF_{2\alpha}$ secretion. The common pathway precursor for regulation of both

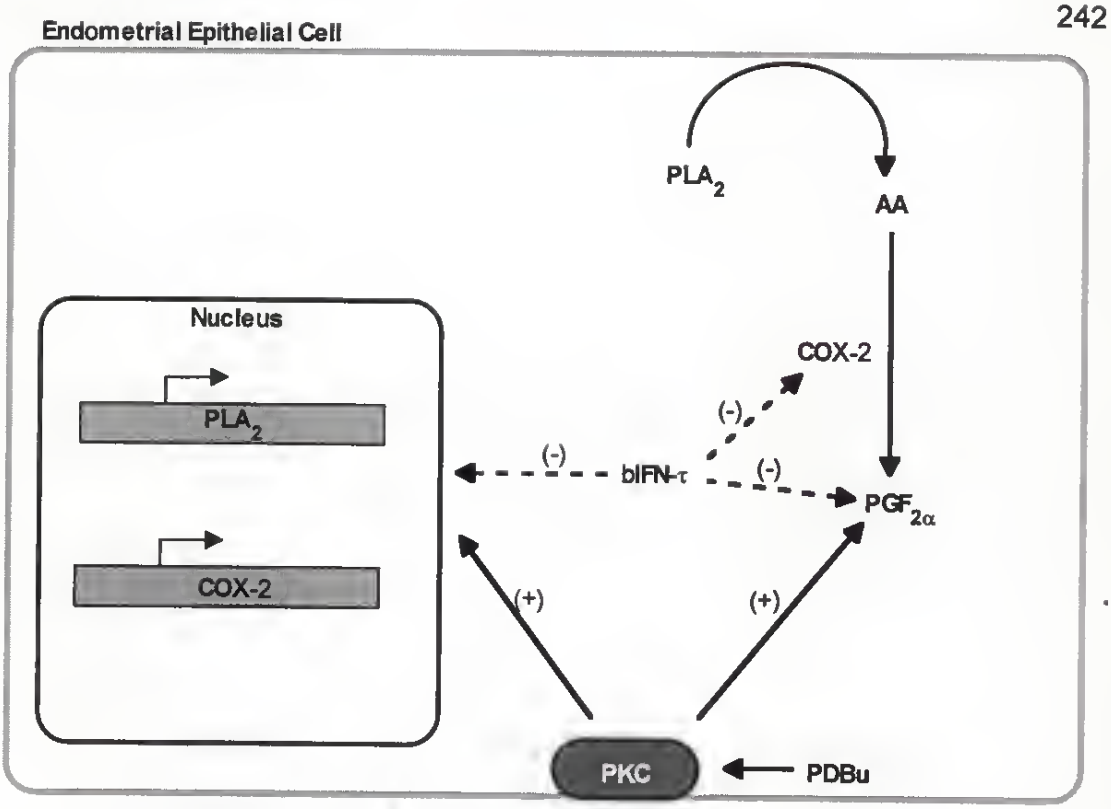


Figure 7-12. Hypothetical model for actions of phorbol 12,13 dibutyrate (PDBu) and bovine interferon-τ (bIFN-τ) in BEND cells. Phospholipase A_2 (PLA_2) cleaves arachidonic acid (AA) from phospholipids in the cell membrane. Cyclooxygenase-2 (COX-2) converts AA into $PGF_{2\alpha}$. The PDBu stimulates protein kinase C (PKC) enzymatic activity, which induced synthesis of both PLA_2 and COX-2 proteins and synthesis of $PGF_{2\alpha}$ was stimulated as a result. Presence of bIFN-τ inhibited protein expression of PLA_2 and COX-2, and COX-2 enzymatic activity to suppress synthesis of $PGF_{2\alpha}$ in BEND cells.

PLA_2 and COX-2 is PKC. Phorbol esters mimic the action of diacylglycerol, a product of phospholipase-C activity, which has the effect of activating PKC activity. The PKC may stimulate $PGF_{2\alpha}$ secretion via stimulation synthesis and/or activity of both PLA_2 (Mayer and Marshall, 1993; Karimi and Lennartz, 1995) and COX-2 (DeWitt, 1991; Vezza et al., 1996). Therefore, effects of bIFN-τ can be at the level of PKC, to inhibit the ability of this enzyme to stimulate $PGF_{2\alpha}$ synthesis through modulation of its mediators, PLA_2 and COX-2. Since not all PDBu-

induced PKC effects were suppressed (i.e., there was still $PGF_{2\alpha}$ production, even in presence of bIFN-τ) it is unlikely that bIFN-τ effects were exerted upstream from PKC (e.g., a block on PDBu ability to bind PKC). It is likely that bIFN-τ blocked some, but not all PDBu effects downstream from PKC.

Regarding the modes of action of bIFN-τ, the fact that attenuation of $PGF_{2\alpha}$ synthesis occurred so quickly suggests that bIFN-τ may act through a pathway alternative to the classical JAK-STAT pathway. The JAK-STAT pathway involves phosphorylation and nuclear translocation of cytoplasmic STAT proteins, as a result of bIFN-τ binding to its receptor (Darnell et al., 1994; Chapter 5). In the nucleus, STAT proteins act as transcription factors to stimulate expression of interferon-stimulated genes. Interferon-induced proteins may act in a variety of ways to produce the interferon-induced phenotype. Presence and function of this classical mode of action in the endometrium has been the prevalent dogma of laboratories studying effects of bIFN-τ on production of $PGF_{2\alpha}$ during maternal recognition of pregnancy in cattle (Austin et al., 1996b; Binelli et al., 1996; Hansen et al., 1997; Perry et al., 1998; Chapter 5 and 6) and sheep (Spencer et al., 1998). For example, ubiquitin cross-reactive protein is induced by bIFN-τ in endometrial explant culture (Austin et al., 1996b; Staggs et al., 1998). This protein forms complexes with other cytosolic proteins, and could modulate their activity and turnover rate (Johnson et al., 1998a). However, complex formation requires 12-hour exposure to bIFN-τ, which makes

UCRP an unlikely mediator of early bIFN-τ suppression of $PGF_{2\alpha}$. Other possible mediators of bIFN-τ actions include the interferon-induced transcription factors IRF-1 and IRF-2 (Harada et al., 1989; Spencer et al., 1998; Chapter 6). IRFs are rapidly synthesized in response to bIFN-τ (detected after 1 hour exposure to bIFN-τ, Chapter 6) and could induce synthesis of specific proteins that could act to suppress $PGF_{2\alpha}$ synthesis. Again, the short time frame of bIFN-τ-mediated suppression of $PGF_{2\alpha}$ makes this possibility questionable. Therefore, I would like to postulate a protein synthesis-independent mode of action for bIFN-τ in the endometrium. Bovine IFN-τ could activate intracellular second messengers other than STAT proteins, which may have modulatory effect on the $PGF_{2\alpha}$ synthesizing machinery. For example, Stancato and others (1997) proposed that binding of interferon α to its receptor can activate the ras-raf pathway, resulting in activation of MAP kinases. Moreover, Pfeffer and coauthors reported a pathway involving PI-3' kinase activation of MAP kinase, stimulated by interferon β. The MAP kinases can modulate a series of intracellular responses and regulation of $PGF_{2\alpha}$ synthesis is a plausible possibility and testable hypothesis. Finally, PLA_2 becomes associated with JAK-1 after stimulation of cells with interferon-α (Flati et al., 1996). It is possible that such an association either sequesters this enzyme or modifies its activity to attenuate its normal function. Collectively, these and other mechanisms may explain protein synthesis-

independent actions of bIFN-τ observed in the present study. Definitive studies with the critical use of protein synthesis inhibitors warrant investigation.

CHAPTER 8
GENERAL DISCUSSION

Distinct from other areas within the greater field of physiology, the study of physiology of reproduction is complicated by the fact that a large number of processes in the reproductive cycle are affected by two beings, the developing conceptus and the conceptus-bearing maternal unit. The immediate consequence of this scenario is that studies in this area must take into consideration not only the individual units involved, but much more importantly, the interactions taking place between the two units. As exemplified in the review of literature (Chapter 2) and further stressed by the specific examples studied in all chapters of this dissertation, successful outcome of the reproductive process is dependent on successful exchange of concerted physiological signals between maternal and embryonic units. This dissertation described examples of communications in the physiological windows of the periestrual period and maternal recognition of pregnancy. To each window there is an associated percentage of embryonic losses in cattle, which calls for further research, if one's goal is to understand causes of such mortality to be able to propose solutions and improve overall pregnancy rates.

There is a much higher percentage of embryonic losses during the window of maternal recognition of pregnancy associated with CL maintenance, compared to during the time gametes/embryos are in the oviduct (5 to 10% vs. 30% respectively, Henricks et al., 1971; Diskin and Sreenan, 1980). This could be attributed simply to the fact that the oocyte/embryos only remain in the oviduct for 72 to 84 hours (Betteridge and Flechon, 1988) vs. 14 days from when they first arrive to the uterus to the time of maternal recognition of pregnancy associated with maintenance of CL. However, I would like to postulate that one main reason for this discrepancy in embryonic losses is associated to the degree of maternal-embryonic interactions occurring in these two periods. In other words, while in the oviduct, there is less exchange of signals occurring (associated with less mortality), it is the opposite in uterus. Although critical studies have not being performed, there is little evidence for embryonic modulation of oviductal function. There is strong evidence for a role of the oviductal environment to affect processes of gamete maturation and transport, fertilization and early embryo development (Gandolfi et al., 1989; Parrish et al., 1989; Anderson and Killian, 1994; Boatman and Magnoni, 1995; Hill et al., 1996; Buhi et al., 1997;), but such processes appear to be vastly regulated through the action of ovarian steroids (Buhi et al., 1991; Murray, 1993), and role of the embryo is negligible. So, although there is communication occurring, it is largely unidirectional. This is in complete contrast with processes occurring in the uterus during maternal recognition of pregnancy associated with the time of CL

maintenance, in which timely exchange of signals between maternal and conceptus units is required for maintenance of pregnancy (Thatcher et al., 1994b; 1997). Such exchange of signals reduces uterine $PGF_{2\alpha}$ secretion (Helmer et al., 1989a; Meyer et al., 1995; Arnold et al., 1999), decreases follicular development (Thatcher et al., 1991) and may support conceptus growth through uterine growth factor production (de Moraes and Hansen, 1997; Paula-Lopes et al., 1998; 1999). These more complex, more highly interactive sets of interactions offer greater opportunity for failure, what could explain the greater percentage of embryo losses during the maternal recognition of pregnancy window associated with CL maintenance at approximately 17 days after estrus.

The fact that a major regulation of oviductal function in exerted through the actions of ovarian steroids, alterations in the endogenous patterns of E_2 and P_4 secretions may have negative implications on fertility. Chapter 3 illustrates modulation of oviductal secretory protein patterns by altered follicular function and steroid secretion. The distinct steroidal milieus conditioned by fresh vs. persistent dominant follicles caused both region and side specific changes in abundance of specific oviductal proteins. Such changes may be associated with the decreased fertility observed in cattle submitted to estrous synchronization protocols that cause formation of persistent dominant follicles (Savio et al., 1993b; Cooperative Regional Research Project, NE-161, 1996). Mechanistically, there could have been changes in critical proteins associated with reproductive processes in the oviduct, such as fertilization and early embryo development.

The fact that pregnancy (Arnold et al., 1999) and bIFN-τ (Meyer et al., 1995) are able to modulate $PGF_{2\alpha}$ secretion from the uterus prompted our laboratory to study the nature of the signal transduction system stimulated by bIFN-τ in the endometrium to exert such an effect. Since bIFN-τ is a type-I interferon, we hypothesized that bIFN-τ activated the classical JAK-STAT pathway and formulated our working hypothesis: binding of bIFN-τ to endometrial receptors activates the JAK-STAT pathway and induces synthesis of bIFN-τ-induced proteins, which function to downregulate the $PGF_{2\alpha}$ synthetic machinery. Data from Chapters 4, 5 and 6 confirmed presence of a functional JAK-STAT pathway in the endometrium (Figure 5-23). Intracellular actions of extracellularly added bIFN-τ suggest the presence of a functional bIFN-τ receptor. Immunoblots confirmed presence, homo- and heterodimer formation and nuclear translocation of STAT proteins. Tyrosine phosphorylation of STAT proteins indicated presence of JAK kinases. Electrophoretic mobility shift assays confirmed biological activity of nuclear localized STATs, which bound to specific DNA sequences present in the regulatory region of interferon-induced genes. Finally, bIFN-τ induced synthesis of intracellular, secretory and nuclear (IRF-1) proteins in endometrial cells and explants. However, data presented in this dissertation does not provide a direct link between activation of JAK-STAT pathway and bIFN-τ -induced suppression of $PGF_{2\alpha}$. However, several possibilities can be considered after examination of results (Chapters 4, 5 and 6)

and reports from other laboratories. For example, putative cis-activating elements for IRFs have been found in the regulatory region of the ovine oxytocin receptor gene (Fleming et al., 1998) and bovine oxytocin receptor gene (Bathgate et al., 1998). Since both receptors have presumed roles in the process of luteolysis, a suppressive effect of bIFN-τ in their expression mediated through IRFs, would be in order with the hypothesis of antiluteolytic effects of bIFN-τ. Test of the hypothesis for a requirement of the JAK-STAT pathway for bIFN-τ -induced suppression of $PGF_{2\alpha}$ will require inactivation of specific components of the JAK-STAT pathway, such as STATs, through use of dominant negative molecules, specific inhibitors, anti-sense technology etc.

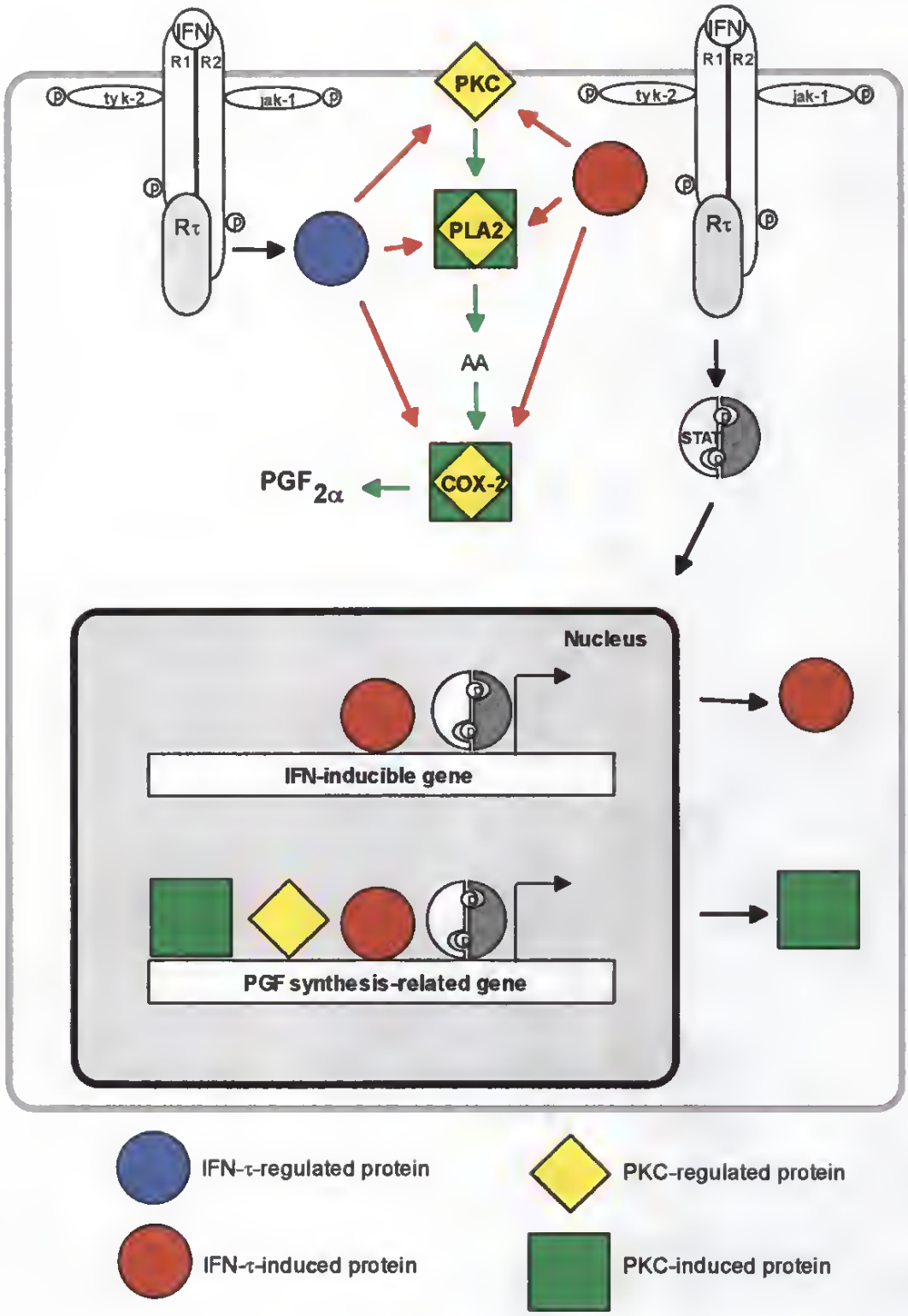
Data from Chapters 5 and 6 provided general information on characteristics of the JAK-STAT pathway in BEND cells, but a number of questions remain unanswered and warrant further investigation. For example, presence of a specific bIFN-τ receptor/signal transduction pathway to decrease $PGF_{2\alpha}$ secretion remains a provoking, largely unanswered question. Data from Staggs and co workers (1998) provide indirect evidence for existence of a bIFN-τ specific receptor/signaling system. They investigated the regulation of endometrial bovine GCP-2 protein secretion and found that although bIFN-τ was able to induce its expression, the closely related bovine interferon-α failed to do so. In contrast, both interferons induced secretion of another endometrial protein, bovine UCRP. Moreover, expression of bovine GCP-2, but not bovine

UCRP could be stimulated by phorbol ester, indicating complex regulation of these proteins. The bovine UCRP gene has five ISREs in its regulatory region, which explains its regulation through the classical JAK-STAT-ISGF-3 pathway (Darnell et al., 1994). However, promoter sequence of bovine GCP-2 has not been published. Collectively, these data indicate that in addition to regulation through the classical JAK-STAT pathway, bIFN-τ may be able to use alternative, specific signaling pathways. For example, binding of bIFN-τ could cause recruitment of a specific STAT protein or another transcription factor, recruitment of a specific polypeptide chain to the receptor complex or of another kinase. Han and Roberts (1998) cloned various forms of type I interferon receptor from ovine and bovine endometrium cDNA libraries and failed to identify novel receptor chains that could be specific for bIFN-τ. They concluded that bIFN-τ utilizes standard IFNαR1 and IFNαR2 for signaling. They used a homology screening method which would not be appropriate to detect a peptide with a unknown sequence (i.e., a novel receptor chain with a unknown sequence). It is my contention that the hypothesis of a unique receptor for bIFN-τ is still open for testing. Additionally, as mentioned above, removal of critical components of the JAK-STAT pathway is an appropriate way to test their requirement for bIFN-τ functional regulation. The bIFN-τ-regulated suppression in PDBu-induced $PGF_{2\alpha}$ secretion model, as described in Chapter 7, would be appropriate to conduct such experiments.

Data from Chapter 7 challenges the concept that protein synthesis is required for bIFN-τ-mediated suppression of $PGF_{2\alpha}$. The quick reduction in the rate of $PGF_{2\alpha}$ accumulation suggests that either bIFN-τ stimulates very quickly synthesis and action of proteins that are inhibitory to the $PGF_{2\alpha}$ cascade, or that bIFN-τ acts in the cytosol to modulate activity of enzymes involved in $PGF_{2\alpha}$ production. This agrees with data from Chapter 4, in which abundance of COX-2 and PLA_2 in endometrial epithelial cells recovered fresh were similar between cows that received uterine infusions of control protein or bIFN-τ. Perhaps modulation of activity of such enzymes, rather than their expression, was critical for bIFN-τ-induced suppression of $PGF_{2\alpha}$. Indeed, intracellular stimulator-induced $PGF_{2\alpha}$ secretion from explants recovered from these same cows was attenuated in cows that received bIFN-τ in vivo. (Arnold et al., 1999). However, critical experiments have not been performed to determine if protein synthesis-independent mechanisms are in place for bIFN-τ suppression of $PGF_{2\alpha}$. Perhaps bIFN-τ stimulates both protein synthesis-dependent and -independent mechanisms to suppress $PGF_{2\alpha}$ synthesis in the endometrium (see Figure 8-1).

Chapter 7 described experiments using a novel model system for study of $PGF_{2\alpha}$ secretion and bIFN-τ effects on $PGF_{2\alpha}$ secretion in the endometrium. Such system features PDBu-induced $PGF_{2\alpha}$ accumulation in culture medium, measurable by radioimmunoassay, and also bIFN-τ inhibition of $PGF_{2\alpha}$

Figure 8-1. Hypothetical model of actions of bovine interferon-tau (bIFN-τ) to prevent protein kinase C (PKC)-induced production of $PGF_{2\alpha}$ in endometrial epithelial cells. Activation of PKC stimulates synthesis (green squares) and enzymatic activity (yellow diamonds) of specific endometrial proteins, such as phospholipase A_2 (PLA_2)and cyclooxygenase-2 (COX-2) to ultimately stimulate synthesis of $PGF_{2\alpha}$. PKC may stimulate synthesis or activity of other cellular mediators, such as kinases, lipases and transcription factors to further enhance synthesis and activity of enzymes directly involved in the $PGF_{2\alpha}$ production. Binding of bIFN-τ to the type I receptor, here represented with a hypothetical, bIFN-τ-specific chain (Rτ), may have protein synthesis-dependent (through activation of the JAK-STAT pathway; right side of diagram) or -independent (left side of diagram) actions to decrease production of $PGF_{2\alpha}$. STAT dimers may translocate to the nucleus to stimulate transcription of interferon-inducible genes and to repress transcription of genes related to synthesis of $PGF_{2\alpha}$ The bIFN-τ-induced proteins (red circles) may act as transcription factors to regulate transcription of genes, or may have cytosolic actions to regulate activity of enzymes involved in production of $PGF_{2\alpha}$. Alternatively, bIFN-τ may regulate activity of pre-existing cellular mediators (blue circles) to negatively affect the $PGF_{2\alpha}$ synthesizing machinery.


IFN
R1
R2
tyk-2
jak-1
Rτ
PKC
PLA2
AA
COX-2
PGF2α
STAT
Nucleus
IFN-inducible gene
PGF synthesis-related gene
IFN-τ-regulated protein
IFN-τ-induced protein
PKC-regulated protein
PKC-induced protein

accumulation. Using this system it was determined that PDBu (probably through stimulation of PKC) stimulates both protein expression and enzymatic activity of PLA_2 and COX-2 in BEND cells. Moreover, bIFN-τ was able to regulate $PGF_{2\alpha}$ secretion also through modulation of protein expression and enzymatic activity of either or both enzymes. Perhaps regulation of PLA_2 activity is a critical feature of bIFN-τ-mediated $PGF_{2\alpha}$ inhibition. In the experiment reported by Arnold and co-authors (1999), explants were obtained from cows that received either bIFN-τ or control protein (bovine serum albumen) infusions in utero. Explants were treated with intracellular stimulators of $PGF_{2\alpha}$ secretion, and $PGF_{2\alpha}$ was measured in culture medium. In vivo treatment with bIFN-τ failed to inhibit $PGF_{2\alpha}$ secretion stimulated by melittin, a PLA_2 activity stimulator. If inhibition of PLA_2 activity is essential for inhibition of $PGF_{2\alpha}$ secretion, it is possible that treatment with melittin overcame bIFN-τ-induced inhibition, resulting in $PGF_{2\alpha}$ production. It is important to note that when endometrium from pregnant vs. cyclic cows were tested for $PGF_{2\alpha}$ secretion in response to the melittin, pregnancy effectively blocked melittin induced $PGF_{2\alpha}$ secretion (Arnold et al., 1999). This supports the notion that bIFN-τ needs to act in concert with other embryonic factors to fully suppress luteolytic mechanisms. Identification of such factors warrants investigation. Studies by Graf and co-authors (1999) and Asselin and co-authors (1997) indicate that regulation of PLA_2 activity in vivo is a preferred hypothesis compared to regulation of gene expression. Abundance of PLA_2 mRNA and

protein did not change during the estrous cycle in sheep (Graf et al., 1999) and bIFN-τ failed to affect PLA_2 gene expression in endometrial epithelial cells (Asselin et al., 1997). Similarly, Boos (1998) monitored changes in immunoreactive COX enzymes (both 1 and 2) in endometrial biopsies obtained from a given animal at days 1, 8, 15 and 19 of the estrous cycle (n=10). There was not a remarkable variation in levels of COX throughout the estrous cycle, also supporting the concept that modulation of activity of COX is associated with $PGF_{2\alpha}$ during luteolysis.

Another intriguing possibility for regulation of $PGF_{2\alpha}$ secretion by bIFN-τ involves fine regulation of PKC activity. The fact that bIFN-τ inhibited PDBu-stimulated $PGF_{2\alpha}$ secretion and PDBu stimulates PKC activity supports the concept of bIFN-τ inhibition of PKC. Since bovine GCP-2 is induced by bIFN-τ (Staggs et al., 1998) and also is stimulated by phorbol esters, part of the bIFN-τ effects could be exerted through PKC. Finally, melittin not only stimulates PLA_2 activity, but also inhibits PKC activity (Gravitt et al., 1994). There could be fine regulation of PKC subtypes to yield differential responses modulated by melittin, PDBu and bIFN-τ.

The endometrium presents a interesting model for studying alternative modes of action of type I interferons. The required down regulation of the $PGF_{2\alpha}$ generation system by bIFN-τ is in variance with effects of other type I interferons in other cell systems. For example, interferon-β stimulates tyrosine

phosphorylation and activity of the serine kinase PI-3' kinase (Pfeffer et al., 1997). This enzyme has been implicated in stimulation of transcriptional activation of COX-2, through activation of PKC activity. Interferons α, β and γ (actually a type II interferon, but that also acts through activation of the JAK-STAT pathway) induce synthesis and activation of PLA_2 (Wu et al., 1994), and PLA_2 was shown to be required for maximum formation of ISGF-3 complex (Flati et al., 1996). Finally, interferon β stimulated PLC activity in human lymphocytes, and this effect was independent of ISGF-3 formation (Miscia et al., 1997). Collectively, these data suggest that either the endometrium has a unique responsiveness to interferons or bIFN-τ modulates the $PGF_{2\alpha}$ secretion machinery differently than other types of interferons. The second hypothesis is less attractive, since early experiments demonstrated that interferon α is able to extend interestrus interval and CL lifespan in dairy cows (Plante et al., 1989).

A question that was asked at the outset of experiments to determine presence and function of JAK-STAT pathway in the endometrium was what is the appropriate experimental model to use (i.e., primary cells, explants etc). Criteria for choice were physiological relevance of a system and convenience of a system for performing intensive, frequent experiments. The system chosen initially was the use of explants, collected from day 15 cyclic cows (Chapter 3). Explant culture is attractive in that structural arrangement of tissue is maintained, allowing critical cell-cell communication to be maintained. Moreover, tissues were collected from specific stages of the estrous cycle, which provided a clear

picture of in vivo events. Also, endometrial explants proved to be a useful system to study secretory and intracellular proteins synthesized de novo in response to bIFN-τ. However, explants proved to be a wrong choice for the study of the JAK-STAT pathway. It was very challenging to detect tyrosine phosphorylation of proteins in explants. This is probably because during the process of homogenization, phosphatases, previously compartmentalized in intact cells, were mixed with phosphorylated STATs. This may have caused removal of phosphate groups, even in the presence of phosphatase inhibitors in the homogenization buffer. Moreover, immunoprecipitation of STATs was only possible after endogenous IgGs were removed by pre-incubation of tissue extracts with protein A-sepharose beads. In the whole tissue extracts, presence of stromal cell proteins diluted epithelial cell proteins, which decreased the ability to detect epithelial cell-specific effects of bIFN-τ. Second in the rank of a physiologically significant model, the use of scraped cells from the endometrial lumen was attempted as described in Chapter 4. This procedure was described by Charpigny and others (personal communication) for sheep endometrium, and presented an attractive alternative to explants, since epithelial cells could be easily isolated (strong cytokeratin staining; Chapter 4), and used immediately, which would minimize loss of in vivo phenotype. This approach would avoid protease digestion, as normally used for preparation of cells for primary culture, minimize loss of membrane receptors and allow immediate use of cells in experiments. Charpigny and co-workers were able to plate cells scraped from

sheep endometrium and analyze synthesis of prostaglandins in relation to COX-2 content. I failed to repeat their technique successfully. After scraping, 99% of bovine endometrial cells were dead, as determined by trypan blue exclusion. Therefore, use of this system for in vitro incubations was not possible with bovine endometrium. However, the system was useful to generate data depicting levels of COX-2 and PLA_2 in endometrium epithelium just after slaughter. These data led us to conclude that similar abundance of COX-2 and PLA_2 in cows receiving control or bIFN-τ uterine infusions indicated that a reduction in $PGF_{2\alpha}$ secretion induced by PDBu may be due to bIFN-τ regulation of their respective enzyme activities. BEND cells provided a model adequate to measure cytosolic and nuclear proteins related to the JAK-STAT pathway (Chapters 5 and 6) and to measure $PGF_{2\alpha}$ secretion and expression of proteins related to the $PGF_{2\alpha}$ secretory machinery (Chapter 7). They originated from day 14 cyclic cows, but how much of the original phenotype is maintained is questionable, and criticism applicable to any cell culture system is also valid here. However, $PGF_{2\alpha}$ secretion regulation by both PDBu and bIFN-τ are maintained. BEND cells do not respond to oxytocin stimulation (Arnold, Badinga, Binelli and Thatcher, unpublished observations) unless they are primed with steroids before oxytocin challenge (Mirando, personal communication). Lack of responsiveness to oxytocin is similar to non-responsiveness in explants from day 15 cyclic cows, challenged with oxytocin (Arnold et al., 1999). Another similarity between explants and BEND cells is that the two systems were responsive to PDBu

treatment. However, while explants responded immediately to PDBu (within 20 minutes; Arnold et al., 1999), BEND cells need at least two hours of exposure to PDBu to start secreting $PGF_{2\alpha}$. This is probably due to the fact that while enzymes needed for $PGF_{2\alpha}$ synthesis are already present in explants, they need to be synthesized and activated in BEND cells. In other words, effects in explants are probably mediated through stimulation of PLA_2 and COX-2 enzymatic activity, whereas in BEND cells, stimulation of both enzymatic synthesis and activity are required. The idea of pre-existing enzymes in explants is corroborated by findings in Chapter 4, showing presence of PLA_2 and COX-2 in freshly scraped endometrial epithelial cells. The bIFN-τ effects in blocking $PGF_{2\alpha}$ secretion in BEND cells should be tested in vitro with the explant system, to further verify the validity of this convenient and physiological cell culture system.

Collectively, antiluteolytic mechanisms exerted by bIFN-τ and pregnancy are not understood completely, but appear to be complex. A fine, ordered and concerted series of events involving multiple signal transduction systems, second-messengers, and mediators, which act to modulate synthesis and activity of enzymes involved in the production of $PGF_{2\alpha}$, probably take place in the endometrium during maternal recognition of pregnancy associated with CL maintenance. Such a series of events are probably stimulated not only by bIFN-τ, but also by other conceptus-derived factors. Breakdown on the conceptus-maternal signaling system during maternal recognition of pregnancy associated

with CL maintenance may explain the large percentage of embryonic loss during this physiological window. Losses can be attributed to a failure of conceptus to send signals, and to failure of the maternal unit to transduce signals that ultimately suppresses synthesis of $PGF_{2\alpha}$. Differences in developmental stages of the conceptus collected at day 17 of pregnancy (critical day for release of antiluteolytic signals; Thatcher and Hansen, 1992) can explain conceptus failures. Conversely, cow to cow variation in ability to extend estrous cycle in response to bIFN-τ treatment (Helmer et al., 1989b; Meyer et al., 1995) explains maternal unit failure. However, whether poor responsiveness to bIFN-τ is consistent for particular cows remains to be determined.

This leads to my final point of discussion. The main justification for the kind of research presented throughout this dissertation is that a better understanding of mechanisms controlling reproduction should lead to better ways to manipulate cow/conceptus physiology to ultimately increase pregnancy rates (i.e., decrease embryonic mortality). An important philosophical question is: should animal scientists be trying to rescue embryos fated to death, due to their own survival incompetence or inadequate maternal environment? Will rescued embryos produce healthy offspring and healthy adult animals? A good assessment of these questions was discussed in Holm and Callesen (1998), where they compared embryos produced in vivo with embryos produced in vitro. During a normal estrous cycle several follicles grow and undergo atresia, but only one ovulates. This "dominant-ovulatory" follicle is considered to be the most

apt to produce offspring. During in vitro production of embryos, oocytes are recovered from a large number of follicles in the ovary, since the ovary usually is sliced with a scalpel blade. In theory, after fertilization of recovered oocytes, this procedure will generate embryos from oocytes that would have never naturally ovulated, perhaps due to incompetence of the oocyte inside. As a result, embryos are being produced and individuals generated that would have never been created if nature was to take its course. Holm and Callensen (1998) summarized data comparing in vivo and in vitro produced embryos. Results are summarized in Table 8-1.

Table 8-1. Differences in pregnancy rates, incidence of congenital defects and birth weight of calves following transfer of in vivo and in vitro produced bovine embryos (Holm and Callesen, 1998)

Parameter		Vitro	Vivo
Pregnancy rates	Fresh (not frozen)	40-50%	50-75%
Abortion rates	>60 days	7-24%	3-5%
Congenial defects	Overall	3-5%	0.5-2%
Birth weights	>50 kg	>30%	<10%

Overall less favorable results characterize in vitro embryo production. This reflects consequences of in vitro production techniques, but may also reflect the lower adequacy of embryos artificially induced to undergo development to term. For example, in vivo derived embryos secreted more bIFN-τ than embryos produced in vitro via a variety of techniques, which may explain partially the lower pregnancy rates presented in Table 8-1 (Stojkovic et al., 1999). Data regarding lifelong productive and reproductive evaluation of animals derived from

in vitro versus in vivo techniques is lacking, but would be crucial to understand long term effects of the in vitro technique. In summary, it is still open to debate the validity of rescuing pregnancies destined to failure. However, with current intensive management systems of high performance animals (e.g., lactating dairy cow) and decreased reproductive performance, modifications of endogenous mechanisms to improve embryonic survival that optimize maternal-conceptus interactions may improve reproductive performance, propagate genetically superior animals and further enhance food production for human consumption. Furthermore, such knowledge may be utilized to regulate reproductive rates in both animals and humans.

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BIOGRAPHICAL SKETCH

Mario Binelli was born March 4, 1968, to Carmem Lucia Rezende de Oliveira and Guilherme Jose Binelli in Rio de Janeiro, Brazil. He is a stepchild to Maria Estela Machado Binelli. He is the oldest of three children. He is married to Eliana Kampf Binelli. In 1990 he received his Bachelor of Science in agronomy from the "Escola Superior de Agricultura Luiz de Queiroz", which is the College of Agriculture in the University of Sao Paulo in Piracicaba, Brazil. From 1990 to 1991 he worked as manager in a dairy and beef farm in Bofete, Brazil. In January 1992 the author enrolled in a master's degree program at the Department of Animal Science at Michigan State University in East Lansing, MI under the supervision of Dr. H. Allen Tucker, and received his title in 1993. He started his PhD program in the Department of Dairy and Poultry Sciences at the University of Florida in August 1994. His career goal is to become a faculty member of a federal or state university in Brazil and to conduct research in the area of animal physiology.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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